Effect of doxycycline on sulfur mustard-induced respiratory lesions in guinea pigs

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SULFUR MUSTARD [SM; bis(2-chloroethyl) sulfide] is an alkylating blistering agent used for chemical warfare in World War I. In many parts of the world, it is now re-emerging as a major threat not only to troops, but also to civilians (18, 19). The injuries consist mainly in epithelial damage to the tissues through which SM is absorbed, i.e., the skin, eyes, and respiratory tract (39, 54) and to other organs such as the liver and kidney with the synthetic MMP inhibitor doxycycline (40 on SM-induced respiratory lesions. They also suggest that doxycycline may hold promise as a therapeutic tool.

Matrix metalloproteinases (MMPs) degrade extracellular matrix components. Several studies have suggested a role for MMPs in the increased vascular permeability and inflammatory cell migration seen in acute lung injury. In a previous study (12), we found elevated levels of MMP-9/92-kDa gelatinase B in bronchial lavage (BL) fluid from guinea pigs 24 h after intratracheal injection of SM; the increase was correlated with the albumin content. MMP-9/92-kDa gelatinase B was found by immunolocalization at epithelial detachment sites. These results suggested a potential role for 92-kDa gelatinase in the genesis of SM-induced epithelial lesions (12).

MMP activities are tightly regulated by natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), which are synthesized and secreted by most connective tissue cells (51), as well as by macrophages (53). However, in disease states, the TIMP increase may not compensate for the much larger increase in MMP-related degradation, as shown in osteoarthritis (48) or atherosclerosis (6). We therefore designed the present study to further investigate the potential role for MMPs in SM-induced respiratory tract epithelial lesions. We used the previously published model of intratracheal administration of SM (10–12). We used immunoblotting to look for TIMP-1 and -2 in BL fluid from guinea pigs exposed to SM, and we show an imbalance between gelatinases and TIMPs by in situ zymography. To further confirm the imbalance between MMP and TIMP and inadequate level of TIMP to counteract increased gelatinase activity, we evaluated the effects of pretreatment with the synthetic MMP inhibitor doxycycline (40) on SM-induced respiratory lesions.

METHODS

Reagents

SM (purity >98%) was obtained from the chemistry department of our institution. SM was stored in ethanol (100 mg/ml) and diluted 1:150 in 0.9% NaCl just before injection. Fluothane was purchased from Laboratoire Belamont (Paris, France). Ethylenediaminetetraacetic acid (EDTA), N-ethyl maleimide, phenylmercuric acetate, and doxycycline were supplied by Sigma Chemical (Saint Quentin Fallavier, France). Polyclonal antibodies to human TIMP-1 were raised in rabbits as previously described (17). Specific rabbit polyclonal antibodies to human TIMP-2 were obtained from Tebu (Le Perray-en-Yvelines, France). Purified human TIMP-1 and human TIMP-2 were a generous gift from Dr. Gillian Murphy (Cambridge Institute for Medical Research, Cambridge, UK). The secondary antibodies used

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for Western blotting were peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako, Trappes, France) diluted 1:1,000.

**SM Administration**

Experiments were performed on male Hartley-strain guinea pigs (Charles River, Saint-Aubin les Elbeuf, France) weighing 250–300 g. Animals were housed in air-filtered temperature-controlled units (21°C) with food and water freely available. SM was administered intratracheally as follows. The guinea pigs were briefly anesthetized with Fluothane via a face mask. After cervicotomy, a drop of 1% lidocaine was instilled subcutaneously. The trachea was gently exposed, and 0.1 ml of either SM (0.2 mg/kg) or solvent (0.5% ethanol in physiological saline) was injected into the trachea through a 24-gauge needle. The cervical incision was closed by two staples. Within 10 min of anesthesia, all guinea pigs had recovered. All injections were performed in a controlled area under a hood by a technician wearing a face mask and butyl gloves, in accordance with the safety regulations of our institution (Centre d’Etudes du Bouchet).

The study was conducted according to American Physiological Society guiding principles for the care and use of animals and was approved by the local ethics committee for animal studies.

**BL and Differential Cell Count**

Guinea pigs were anesthetized with pentobarbital sodium (50 mg/kg ip) and bled via section of the aorta. After en bloc excision of the heart and lungs, BL was performed through the trachea with nine 2.5-ml instillations of sterile, pyrogen-free 0.09% saline. As in our previous study, fractional processing of BL fluid (12) was used to enrich the samples in bronchial material. The first 2.5-ml aliquot representing the bronchial portion of the fluid (BL) was collected separately. Total and differential cell counts and separation of BL fluids from cells were performed as previously described (12). BL fluids were frozen at −80°C until use. BL fluid proteins were measured according to the method of Bradford (8).

**TIMP Immunoblotting**

Aliquots of BL fluids were separated by SDS-PAGE and transferred to an Immobilon-P filter. Nonspecific sites were blocked by incubating the membranes for 90 min at 10 mM Tris·HCl buffer (TBS), pH 7.40, containing 150 mM NaCl, 5% nonfat dried milk, and 0.05% Tween 20. The transfers were then incubated overnight with polyclonal rabbit anti-human TIMP-1 or TIMP-2, diluted 1:200 in TBS. Horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins were used as a positive control, and the absence of reactivity in the absence of primary antibody was also checked.

**Gelatinase Activity**

Gelatinase activity was evaluated both by zymography and by degradation of a biotinylated substrate. During zymography, the dissociating conditions dissociate active MMP-TIMP complexes, so that the result indicates “total” gelatinase activity. In contrast, degradation of a biotinylated substrate measures “free” activity due to active MMPs not complexed to TIMP, i.e., to “excess” MMPs. For zymography, aliquots of BL fluid were subjected to electrophoresis on polyacrylamide gels containing 1 mg/ml gelatin in the presence of SDS under nonreducing conditions, with one lane per animal. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 1 h, rinsed briefly, and incubated for 24 h at 37°C in a buffer containing 100 mM Tris·HCl and 10 mM CaCl₂, pH 7.40. After incubation, gels were stained with Coomassie brilliant blue R250 and destained in a solution of 7.5% acetic acid and 5% methanol. Zones of enzymatic activities were indicated by negative staining: proteolytic areas appeared as clear bands against a blue background.

Measurement of gelatinase activity by degradation of biotinylated substrate was performed using the MMP gelatinase assay kit (Chemicon, Temecula, CA) as recommended by the manufacturer. The value in each sample was compared with standard curves obtained with various amounts of purified MMP-2 and expressed as a percentage of activity, as recommended by the manufacturer. Inhibition by EDTA (2 mM) was used to ensure that activity was due to MMP.

**In Situ Zymography**

We have used this method previously to assess net proteolytic activity in situ in tissue sections (50). In brief, frozen tracheal sections were applied to uncoated glass slides and allowed to reach room temperature. The glass slides holding the sections were dipped into a photographic emulsion (NTB-2; Kodak, Paris, France) that was diluted 1:3 with distilled water. Emulsion-covered slides were placed horizontally in humidified chambers and incubated for 24 h at 37°C. At the end of incubation, the emulsion was allowed to dry, and the slides were processed by photographic development using D19 developer (Kodak) and then examined under a transmitted light microscope. The background appeared black as a result of emulsion exposure to ambient light during incubation and processing. Zones of gelatin lysis were white against this black background.

**Histology**

Guinea pigs were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium, and a catheter connected to a fixative container was inserted into the trachea via a ventral incision in the neck. Simultaneously, the animals were exsanguinated by section of the abdominal aorta. The thorax was opened, and the lungs were immediately inflated in situ through the tracheal cannula with 4% formaldehyde in cacodylate buffer, pH 7.2, at a pressure of 25 cmH₂O. The trachea was then removed and immersed in fixative for 8 days. Three samples of the lower third of the trachea were further processed for embedding in Paraplast Plus (Gassalem, Paris, France). Sections 5 μm in thickness were stained routinely with hematoxylin-phloxin-saffron. All observations were done by a person who was unaware of the treatment given to the guinea pigs.

**Experimental Protocol**

**Evaluation of the imbalance between gelatinase and TIMP.** BL was performed in two separate experiments, each of which included seven guinea pigs given SM intratracheally and seven given solvent (1% ethanol in PBS). BL was done 24 h after intratracheal instillation of SM or solvent. Cell counts, gelatin zymography, protein assay, and immunoblotting were performed on BL fluids.

**Effect of doxycycline pretreatment.** Effects of doxycycline were tested in three separate experiments, each including six guinea pigs per group. Guinea pigs were pretreated with a subcutaneous injection of doxycycline (30 mg/kg) or 0.9% saline 3 h before intratracheal instillation of either SM or 2% ethanol in 0.9% saline. Subsequently, four groups of guinea pigs were established: a group given 0.9% saline, subcutaneously then ethanol intratracheally (control group), a group given doxycycline subcutaneously then ethanol intratracheally (doxycycline group), a group given 0.9% saline subcutaneously then SM intratracheally (SM group), and a group given doxycycline subcutaneously then SM intratracheally (doxycycline-SM group). The guinea pigs were killed 24 h after the intratracheal injection. Cell counts, total gelatinase activity, free gelatinase activity, and protein and albumin levels were determined in BL fluids. Additional animals in each group were used for histology and in situ zymography.
Statistical Analysis

Data are presented as the means ± SE. Results in the control and in the SM-exposed groups were compared by the nonparametric Mann-Whitney U-test. Data were analyzed with a personal computer using the statistical software package Statview (version 5.0, Abacus Concept; SAS Institute, Cary, NC). P values < 0.05 were considered significant.

RESULTS

Evaluation of the Gelatinase-TIMP Imbalance

Cytology, protein content, TIMP immunoblotting. The total number of cells recovered from BL fluids was considerably higher in SM-exposed guinea pigs than in controls (0.85 ± 0.16 × 10^6 and 0.41 ± 0.05 × 10^6 cells, respectively; P < 0.05). SM exposure significantly raised the protein level in BL fluids (3.0 ± 0.6 and 0.6 ± 0.5 g/l, respectively; P < 0.05).

Western blots of BL fluid with anti-TIMP-1 antibodies showed a 28-kDa band corresponding to TIMP-1. The amount of TIMP-1 was similar in SM-exposed animals and controls, except in one animal (Fig. 1, top). Similarly, Western blots of BL fluid with anti-TIMP-2 antibodies showed a 20-kDa band corresponding to TIMP-2, with no difference between SM-exposed guinea pigs and controls (Fig. 1, bottom).

Gelatinase activity in BL fluids. Gelatin zymograms of control BL fluids (Fig. 2A) showed small amounts of MMP-2/72-kDa gelatinase, mainly in the active 68-kDa form, with low levels of pro-MMP-9/92-kDa gelatinase activity. SM exposure was associated with elevations in total gelatinolytic activities, mainly pro-MMP-9 and active MMP-9, in keeping with our earlier results (12). Free gelatinolytic activity, related to active gelatinase not complexed to inhibitors, was significantly higher in the group exposed to SM than in the controls (Fig. 2B), confirming the imbalance between gelatinases and inhibitors.

In situ zymography. In situ zymography demonstrated low levels of gelatinolytic activity in tracheal epithelium from control guinea pigs. By contrast, and although in situ zymography is not a quantitative method, gelatinolytic activities

Fig. 1. Top: identification of tissue inhibitor of matrix metalloprotease (TIMP)-1 by Western blot in bronchial lavage (BL) from guinea pigs 24 h after intratracheal injection of ethanol (controls) or sulfur mustard (SM). Each lane corresponds to BL from 1 guinea pig (left: controls; right: SM-exposed animals). The 28-kDa band was identified as TIMP-1. No differences between controls and SM-exposed guinea pigs were detected. Bottom: identification of TIMP-2 by Western blot in BL from guinea pigs 24 h after intratracheal injection of ethanol (controls) or SM. Each lane corresponds to BL from 1 guinea pig (left: controls; right: SM-exposed animals). The 21-kDa band was identified as TIMP-2. No differences between controls and SM-exposed guinea pigs were detected.

Fig. 2. Gelatinase activity in BL fluids assessed by gelatin zymography (A) and by degradation of a biotinylated substrate (B). A: gelatin zymogram of BL fluid from guinea pigs 24 h after intratracheal instillation of ethanol or SM. BL fluids (10 μl/well, with each well representing a different animal) were subjected to electrophoresis on SDS substrate gel containing 1 mg/ml of gelatin. After migration, the gels were incubated for 24 h in Tris buffer, stained with Coomassie blue, and destained in 7.5% acetic acid-5% methanol solution. White lytic zones represent gelatinolytic activity. On the left, molecular masses in kDa of major species of gelatinases are given. Gelatin zymograms from control guinea pigs showed small amounts of MMP-2 and MMP-9 gelatinases, whereas larger amounts of both gelatinases were detected in SM-exposed guinea pigs. B: increase in free gelatinase activity in BL from SM-exposed guinea pigs compared with controls. Results are expressed as the relative activity (%) in a control obtained with known amounts of MMP-2, as recommended by the manufacturer. *Significant difference.
appeared considerably increased in trachea from SM-exposed animals (Fig. 3).

**Effect of Doxycycline Pretreatment**

*Inhibition of gelatinolytic activities.* Doxycycline pretreatment was associated with a decrease in gelatinolytic activity, as demonstrated by SDS-PAGE zymography of BL fluids (Fig. 4), which showed decreases in both MMP-2/72-kDa gelatinase and MMP-9/92-kDa gelatinase.

*Cytology and protein content.* Doxycycline pretreatment induced a marked decrease in total-cell and neutrophil counts in BL (Fig. 5, *top* and *middle*). The protein level in BL from SM-exposed animals was significantly lower after doxycycline pretreatment (Fig. 5, *bottom*).

*Histology.* Compared with controls (Fig. 6A), SM-exposed animals showed vacuolar degeneration and detachment of ciliated cells (Fig. 6B) leading in some places to the development of bullae between degenerating cells and basal cells, as previously described (12). Subepithelial edema with inflammatory cell infiltration was observed in the most severe epithelial lesions. Complete epithelial cell detachment was seen in some places. When guinea pigs were pretreated with doxycycline, no bullae and only minimal vacuolar degeneration were found in the epithelium, with no epithelial cell detachment (Fig. 6C) or subepithelial edema.

**DISCUSSION**

The gelatinase-TIMP imbalance shown in our study further supports a role for gelatinases in the respiratory lesions observed after SM exposure, in keeping with our earlier findings (12). To further investigate the role for insufficient level of TIMP in SM-induced respiratory lesions, we studied the effect of doxycycline, an MMP-inhibiting tetracycline. Treatment of guinea pigs with doxycycline before SM exposure significantly protected against the respiratory lesions usually observed after SM exposure. In aggregate, our results support the hypothesis that MMPs play a role in SM-induced respiratory lesions and that TIMP levels are inadequate to counteract increased MMPs. They also suggest that tetracyclines may hold promise as a prevention for SM-induced respiratory damage.

TIMPs, which are naturally occurring MMP inhibitors, are produced by many cell types in cultures and are found in body fluids and tissue extracts (51). They may contribute to regulate various normal MMP-related degrading processes, for instance during morphogenesis and growth (36). An imbalance in the relative concentrations of MMPs and TIMPs has been reported in many diseases, including arthritis, atherosclerosis, tumor growth, and metastasis development (20, 21). In our study, several findings indicate an imbalance between MMPs and TIMPs in SM-exposed guinea pigs: 1) MMP gelatinase activity evaluated by zymography was higher in BL fluid from SM-exposed guinea pigs than from controls, whereas levels of...
TIMP-1 and -2 evaluated by immunoblotting were similar in the two groups; 2) free gelatinolytic activity was increased in BL fluids from SM-exposed guinea pigs compared with controls; and 3) extensive areas of lysis were shown by in situ zymography, indicating net proteolytic activity in situ. These results support a potential role for gelatinases in SM-induced respiratory lesions, in keeping with our earlier study (12), and insufficient levels of TIMP to counteract this increase. A role for proteolytic activity in SM-induced lesions in organs other than the lungs has been suggested by Cowan et al. (14), who found that cytotoxic doses of SM caused increases in serine-proteinase activities of human peripheral blood lymphocytes after in vitro SM exposure, as assessed with synthetic substrates. These authors found a similar serine-proteinase increase in SM-damaged skin of hairless guinea pigs compared with controls decreased significantly with doxycycline pretreatment (bottom). *Significant differences.

Despite these reports of increased protease activity after SM exposure, the effects of SM on protease inhibitors had not been studied previously, whereas both MMPs and their inhibitors should be evaluated, as both may be increased in some disorders. We found an imbalance between MMPs and their inhibitors, with an excess of gelatinases. To further confirm the role for the imbalance between MMP and TIMP in SM-induced respiratory lesions, we investigated the effects of doxycycline, an MMP inhibitor. We chose subcutaneous route of administration as previously published (26, 46).

In the present study, doxycycline pretreatment was associated with 1) a decrease in gelatinase activity as evidenced by zymography, free gelatinase activity, and in situ zymography; 2) a decrease in inflammatory processes as assessed by BL cellularity; and 3) a dramatic decrease in epithelial lesions in to SM (32), with predominant destruction of laminin and type IV collagen in the basal membrane. Our results are also in accordance with the finding by Sabourin et al. (42), that mRNA for MMP-9 but not MMP-2 increased after SM exposure in a model of weanling pig skin.

Fig. 5. Quantification of cells in BL fluid recovered 24 h after ethanol (control) or SM intratracheal injection. In SM-exposed animals, the total cell count (top) was lower with than without doxycycline pretreatment. Neutrophils, the cell type accounting for most of the post-SM increase, decreased significantly as a percentage of total cells with doxycycline pretreatment (middle). BL protein content increased almost 6-fold in SM-exposed guinea pigs compared with controls decreased significantly with doxycycline pretreatment (bottom). *Significant differences.

Fig. 6. Representative light micrographs of tracheal sections stained with hematoxylin-phloxin-saffron showing histological lesions induced in guinea pigs by intratracheal SM injection 24 h earlier. A: trachea of a control guinea pig. In B, after SM exposure, subepithelial edema (arrowhead c) with inflammatory cell infiltration (arrowhead b), vacuolization of epithelial cells (arrowhead a), and intraepithelial clevage (*) are visible. Doxycycline pretreatment 3 h before SM exposure (C) prevented the histological tracheal lesions: there was no edema or intraepithelial clevage. The bar represents 50 μm.
histological specimens. This reduction in SM-induced respiratory lesions observed after doxycycline pretreatment further supports a major role for MMPs. Doxycycline has been reported to inhibit the activity of both 72-kDa (MMP-2) and 92-kDa (MMP-9) (37, 47), but also collagenases (MMP-1, MMP-8, and MMP-13) (27, 45) and matrilysin (MMP-7) (22). The mechanisms by which doxycycline causes MMP inhibition are not fully understood. Doxycycline binds directly to Zn\(^{2+}\) or Ca\(^{2+}\) associated with the MMP molecule, blocking the active site. It can also inhibit activation by other MMPs and also induce conformational changes that render the proenzyme susceptible to fragmentation during activation (25). Inhibition of the transcription of MMP mRNAs has also been reported. In the present study, we observed a decrease in gelatinase activity measured by zymography. Given that doxycycline probably became dissociated from MMP during zymography, a reduction in activity on the zymogram gel may indicate that less enzyme was present in the BL fluid. This suggests that the mechanisms leading to lowered MMP activity may be related either to inhibition of the transcription of MMP mRNAs or to degradation of pro-MMP zymogen during extracellular activation. We also found decreases in the amount of active forms of MMP-9/92-kDa gelatinase, indicating that doxycycline possibly prevented the in vivo activation of pro-MMP zymogens by reactive oxygen species. Petrinec et al. (40) showed that doxycycline limited the development of elastase-induced abdominal aortic aneurysm in a rat model. In this model, increased local MMP production is among the potential mechanisms of structural protein degradation. Doxycycline suppressed the increased production of MMP-9/92-kDa gelatinase by infiltrating macrophages, whereas the production of 72 kDa was unaffected. In our model of SM exposure, the increase was greatest for MMP-9/92-kDa gelatinase, which seemed responsible for the lesions, in keeping with our earlier study (12). In addition, our results suggest that MMP-9/92 kDa may originate not only from inflammatory cells, but also from epithelial cells, in keeping with in vitro results in a previous study (12). Interestingly, doxycycline partially inhibited the recruitment of inflammatory cell recruitment but almost completely prevented the histological lesions, further underlining the importance of local MMP-9 production by epithelial cells. However, doxycycline is not specific for MMP-9, and effects on other possibly increased MMPs cannot be ruled out. Tetracyclines have recently been found effective in treating non-SM-induced inflammatory respiratory tract lesions, reducing airway inflammation and hyperresponsiveness in a murine model of toluene diisocyanate-induced asthma (29) and diminishing acute lung injury after cardiopulmonary bypass (13).

Doxycycline was used in the present study primarily to support the hypothesis that increased MMP gelatinase and inadequate levels of TIMP were involved in the development of respiratory lesions in response to SM exposure. However, the preventive effect of doxycycline also suggests a therapeutic role. The prevention and treatment of SM toxicity are currently a focus of active research. Two main approaches have been suggested for counteracting the toxic effects of SM: application of topical skin protectants (TSPs) and administration of systemic medications. TSPs can prevent both the cutaneous and the systemic toxicity of SM. Indeed, SM is a highly reactive compound that diffuses rapidly to the systemic circulation (35), inducing lesions at a distance from the contact site, for instance in the liver and immune system. Several TSPs were approved by the U.S. Food and Drug Administration in 1999 (9). However, TSPs are not expected to be effective in treating established respiratory lesions. Systemic treatments for SM toxicity are either anti-inflammatory drugs or free-oxygen-radical scavengers. Other compounds likely to protect against alkylating effects have also been tested; an example is synthetic aminothiol amifostine, which is used as a chemical radioprotector. In experiments conducted in vitro on rat liver slices and in vivo, amifostine decreased hepatic lesions when administered 30 min before SM, but the effect on respiratory lesions was not reported (7).

Several antioxidants have been evaluated in vitro and exhibited protective effects on the respiratory cell line A549 (30, 31, 33). In vivo, N-acetyl cysteine delivered by intratracheal instillation simultaneously with SM decreased the inflammatory response to SM, i.e., the influx of neutrophils (4). More recently, various antioxidants were tested in animals exposed to SM by inhalation or percutaneously. In this study, trolox and quercetin increased survival time after SM exposure and decreased oxidative damage to lung tissue (28). Most of the anti-inflammatory drugs tested for their SM-protective potential are glucocorticoids (2, 3, 5). There is also one report of a protective effect of L-nitroarginine methyl ester on chick embryo forebrain neurons in vitro (43). No in vivo studies of arginine analogs have been reported to date.

Interestingly, doxycycline combines all these effects. In addition to inhibiting MMP, doxycycline may also inhibit nitric oxide (NO) production and scavenge free oxygen radicals. Indeed tetracyclines decrease inducible nitric oxide synthase (iNOS) expression and destabilize iNOS mRNA in macrophages and mesangial cells (1), decrease the production of tumor necrosis factor-\(\alpha\) (23), and scavenge reactive oxygen species (52). The decrease in NO and in reactive oxygen species may in turn contribute to further diminish MMP levels, as both systems are known to promote activation of several pro-MMP zymogens. The pluripotent spectrum of doxycycline ant-inflammatory activity may be of special interest in SM-induced lesions, as these are characterized by an intense inflammatory reaction, with increases in iNOS (38), production of nitrates (44), and oxidative stress (24, 34), which are probably involved in the toxicity of alkylating agents.

In conclusion, the present study demonstrates an imbalance between MMPs, most notably gelatinases, and their tissue inhibitors, TIMPs, in SM-induced respiratory lesions. This imbalance supports the use of MMP inhibitors to prevent or treat SM-induced toxicity. Doxycycline pretreatment partially prevented inflammatory cell recruitment and almost completely normalized tracheal histology. Tetracyclines, most notably chemically modified tetracyclines devoid of antibiotic effects, may hold valuable therapeutic potential although animal models have limitation compared with human disease. Studies are under way to optimize the administration route and to test the effects of administration after SM exposure.

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GRANTS

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