MolecularMedicine

Midkine Is Expressed and Differentially Processed during Chronic Obstructive Pulmonary Disease Exacerbations and Ventilator-Associated Pneumonia Associated with *Staphylococcus aureus* Infection

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Staphylococcus aureus is sometimes isolated from the airways during acute exacerbations of chronic obstructive pulmonary disease (COPD) but more commonly recognized as a cause of ventilator-associated pneumonia (VAP). Antimicrobial proteins, among them midkine (MK), are an important part of innate immunity in the airways. In this study, the levels and possible processing of MK in relation to *S. aureus* infection of the airways were investigated, comparing COPD and VAP, thus comparing a state of disease with preceding chronic inflammation and remodeling (COPD) with acute inflammation (that is, VAP). MK was detected in the small airways and alveoli of COPD lung tissue but less so in normal lung tissue. MK at below micromolar concentrations killed *S. aureus in vitro*. Proteolytic processing of MK by the staphylococcal metalloprotease aureolysin (AL), but not cysteine protease staphopain A (SA), resulted in impaired bactericidal activity. Degradation was seen foremost in the COOH-terminal portion of the molecule that harbors high bactericidal activity. In addition, MK was detected in sputum from patients suffering from VAP caused by *S. aureus* but less so in sputum from COPD exacerbations associated with the same bacterium. Recombinant MK was degraded more rapidly in sputum from the COPD patients than from the VAP patients and a greater proteolytic activity in COPD sputum was confirmed by zymography. Taken together, proteases of both bacteria and the host contribute to degradation of the antibacterial protein MK, resulting in an impaired defense of the airways, in particular, in COPD where the state of chronic inflammation could be of importance.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2013.00045

INTRODUCTION

The airways have constitutive host defense mechanisms including mucociliary clearance, alveolar macrophages sensing potential pathogens, and antimicrobial host defense proteins (HDPs) with a potency to eradicate bacteria (1). In addition to their antibacterial activity, HDPs may act as growth factors and regulate leukocyte trafficking (2). Several HDPs are constitutively produced by airway epithelial cells to maintain host integrity, although many are induced by inflammation or stored preformed in innate immune cells (for example, LL-37 in neutrophils). Recently, we found that the growth factor midkine (MK) has potent bactericidal and fungicidal activities (*in vitro*) executed through disruption of microbial membranes, similar to the activity exerted by other innate antibiotics such as the human β-defensins (hBDs), LL-37 and antibacterial chemokines (1,3,4). MK is constitutively expressed during healthy conditions in several tissues, for example, by epithelial cells in the large airways and by keratinocytes in the skin, where it may reach bactericidal concentrations in the microenvironment and thus contribute to host defense (5,6). Several factors present at sites of inflammation promote MK expression including retinoic acid (RA), hypoxia, reactive oxygen species and activation of the transcription factor nuclear factor (NF)-κB (7–11).

Staphylococcus aureus is an important human pathogen. Although uncommonly

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Submitted May 13, 2013; Accepted for publication September 11. 2013; Epub (www.molmed.org) ahead of print September 12, 2013.

associated with respiratory tract infections, it is sometimes isolated from sputum during acute exacerbations of chronic obstructive pulmonary disease (COPD), and it is a relatively common cause of hospital-acquired and, in particular, ventilator-associated pneumonia (VAP). *S. aureus* is estimated to account for 10–20% of all nosocomial pneumonias (12). The reported mortality in VAP patients ranges from 33% to 72%, with the upper range reflecting the increased risk for mortality among the elderly, patients with impaired cardiopulmonary function, immune compromised patients and patients who require prolonged intubation of the airways. *S. aureus* has a plethora of countermeasures against host defense mechanisms, including surface proteins and secreted proteases (13). The continuing increase in antibiotics resistance among *S. aureus* strains, and in particular the spread of meticillin-resistant *S. aureus* (MRSA), constitute new challenges for health care and health care professionals (14).

The aim of this study was to investigate the levels and possible processing of MK in relation to *S. aureus* infection of the airways, comparing COPD and VAP, with the former being characterized by a state of chronic inflammation and remodeling, whereas in VAP, the airways respond with an acute inflammation. We found expression and location of MK within COPD airways. During infection with *S. aureus*, MK was undetectable in COPD sputum, but high levels were detected in aspirations from VAP patients. Staphylococcal proteases fragmented MK and thereby affected its bactericidal activity. COPD sputum showed a higher proteolytic activity than VAP aspirations. We believe this to be an important example of defense impairment in the airways during COPD.

MATERIALS AND METHODS

Patient Groups and Samples

In the present study, lung tissue from 11 COPD (Global Initiative for Chronic Obstructive Lung Disease [GOLD] stage IV) patients was used (Table 1). In addition, healthy lung tissue from nine nonsmoking controls was obtained during lung lobectomy because of suspected lung cancer in otherwise healthy nonatopic individuals. Only patients with solid tumors with visible borders were included in the study, and tissues used were obtained as far from the tumor as possible. In patients with very severe COPD (GOLD IV), matching lung tissue was collected in association with lung transplantation. For all patient groups, care was taken to immerse the tissue into fixative immediately after surgical excision, and multiple large tissue blocks were prepared for histological analysis. All subjects gave written informed consent to participate in the study, which was approved by the local ethics committee in Lund, Sweden (LU412-03).

Table 1. Characteristics of patients with COPD and healthy controls whose tissue was used for histological analysis.

	Controls	COPD GOLD IV
Sex (M/F, n)	2/7, 9	5/6, 11
Age[a] (years)	63 (33–76)	58.5 (53–66)
Pack-years[a] (years)	0	40.7 (2560)
Exsmokers/current smokers	0	11/0
GCS (yes/no/unknown)	0	10/0/1[b]
β_2 Agonist (yes/no/unknown)	0	10/0/1[b]
Lung function		
FEV_1[a]	2.7 (1.7–5.1)	0.6 (0.4–1.0)
FEV_1/(F)VC[a]	85.9 (66–121)	30.2 (17–39)
FEV_1 % of predicted[a]	109.8 (82–141)	21.4 (13–27)

FEV_1, forced expiratory volume; (F)VC, (forced) vital capacity; GCS, inhaled glucocorticosteroids.
[a]Data are given as mean (range).
[b]n = 11; one subject with missing medical history.

Sputum from COPD patients and endotracheal aspirations from 20 consecutive patients diagnosed with VAP (both groups had positive cultures for *S. aureus*) were collected at Skåne University Hospital between August 2011 and January 2012 and stored at –20°C until analysis. The study design was a cross-sectional study of patients suffering from acute and chronic airway inflammatory responses, respectively, as represented by patients with *S. aureus*–associated VAP or COPD exacerbation. Intervention was collection and culture of sputum or tracheal aspirates upon signs of airway infection/exacerbation. Power analysis assuming a difference between means of 2 ng/mL and 1 standard deviation (SD) indicated that six patients in each group were required to achieve a power of 0.8 at $\alpha = 0.05$. Patients included in other trials (n = 3) or where sputum sample was too viscous (n = 3) were excluded from all parts of our study.

In patients suffering from VAP, endotracheal aspirates were collected by using a sterile suction catheter via an endotracheal tube or a tracheostoma. In patients suffering from COPD, sputum was mobilized using the huffing technique, without inhalation of nebulized sodium chloride. Parts of the samples were used for culture and parts were frozen at –20°C until use in assays. After thawing, sputum was centrifuged at 5,000*g* for 5 min and aliquots of the supernatant were used. Sputum was used without addition of dithiothreitol to make it possible to use antibody-based measurement of MK (that is, enzyme-linked immunosorbent assay [ELISA]). The protocol was approved by the ethics committee at Lund University (#492/2007).

Immunohistochemistry

Immediately after collection, tissues were placed in 4% buffered formaldehyde, dehydrated and embedded in paraffin, and thin sections (3 μm) were generated.

Single staining of MK. A single staining protocol (EnVision™ Detection system, K5007; Dako, Glostrup, Denmark)

was used for visualization of MK. Briefly, MK was detected by a rabbit anti-MK antibody (Peprotech, London, UK), and secondary antibodies were conjugated with peroxidase polymers. The immunohistochemistry protocols were performed by using an automated immunohistochemistry robot (Autostainer; Dako). Sections were stained with Mayer's hematoxylin for visualization of background tissue and were dehydrated in alcohol and xylene and mounted in Pertex (Histolab).

Double staining of MK and surfactant protein A (SP-A) or myeloperoxidase (MPO). After antigen retrieval and a blocking step (protein-blocking, ×0909; Dako), sections were incubated overnight in 4°C with a rabbit anti-MK antibody, and immunoreactivity was visualized after 1-h incubation at room temperature (RT) with a goat anti-rabbit Alexa Fluor 555–conjugated secondary antibody (1:200; Molecular Probes/Life Technologies, Carlsbad, CA, USA). SP-A was detected after 1-h incubation at RT with a mouse anti-SPA (1:50; Dako) and visualized after incubation at RT for 1 h with an Alexa Fluor 488–conjugated goat anti-mouse secondary antibody (1:200; Molecular Probes/Life Technologies). Consecutive sections were stained with a directly conjugated rabbit anti-MPO (1:12,000; Dako) by using a Rabbit Alexa Fluor 488 nm Zenon labeling kit according to the manufacturer's instructions (Molecular Probes/Life Technologies). Nuclei were detected by Hoechts 33342, and sections were mounted in TBS/glycerin and frozen until quantification. For all immunohistochemical procedures, markers and tissues, staining was absent in sections using isotype-matched control antibodies (Dako) that were used instead of, and at the same concentration as, the primary antibody.

Bacterial Strains, Determination of Antibiotics Resistance Profile and Screening of Virulence-Associated Genes

The *S. aureus* strains 5120, Newman, 8325-4 and 8325-4 sarA (sarA; deficient of the repressor sarA, resulting in overexpression of proteases released extracellularly [15]) were used for the *in vitro* studies and routinely cultured in Todd-Hewitt medium (TH; BD, Franklin Lakes, NJ, USA) at 37°C with 5% CO_2.

The antibiotics susceptibility of the *S. aureus* strains isolated from COPD and VAP patients were tested by using the disc diffusion method according to the Swedish Reference Group for Antibiotics (SRGA) and its subcommittee on methodology (SRGA-M; www.srga.org) or by E-test (bioMeriéux, France). Discs with cefoxitin, clindamycin, fusidic acid, trimethoprim-sulfamethoxazole, norfloxacin, tigecyclin, gentamicin, linezolid and rifampicin were obtained from Oxoid (Basingstoke, UK). The vancomycin susceptibility was tested using E-tests. All isolates were screened for the presence of virulence-associated genes encoding Panton Valentine leucocidine (*lukS*-PV and *lukF*-PV) and *tss* encoding the TSS-1 toxin using polymerase chain reaction and *spa* typed as previously described (16).

Bactericidal Assay

Bacteria was cultured to the midexponential growth phase ($OD_{620} \approx 0.4$), washed in incubation buffer (10 mmol/L Tris/HCl, 5 mmol/L glucose; pH 7.5) and adjusted to a concentration of 2×10^6 colony-forming units (CFUs)/mL. Fifty microliters bacterial solution (~10^5 CFUs) was incubated in buffer or with various concentrations of the indicated protein or mixtures for 1 h at 37°C. To quantify the bactericidal activity, serial dilutions in incubation buffer were plated on TH-agar in duplicates and incubated overnight at 37°C. The percent bacterial killing was calculated by comparison with the number of CFUs when incubated in buffer alone.

Electron Microscopy

Bacteria were incubated with MK (1 μmol/L) in incubation buffer for 1 h at 37°C and then subjected to scanning electron microscopy as described (17). Immunogold labeling and transmission electron microscopy (TEM) of ultrathin sections was performed as previously described (5). Specimens were examined in a JEOL (Tokyo, Japan) 1200EX transmission electron microscope operated at 60 kV accelerating voltage.

Protein Detection by ELISA, Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

The MK ELISA was from Peprotech. For Western blotting, standard techniques were used. After separation on a Tris-tricine gel, samples were electroblotted onto a polyvinylidene fluoride membrane. After blocking, membranes were probed with antibodies toward MK (Peprotech), followed by a secondary goat–anti-rabbit antibody (Bio-Rad, Hercules, CA). Blots were developed by using the Bio-Rad Chemidoc system (Bio-Rad).

MK Proteolytic Cleavage Assay

MK (5 μg) was incubated for the indicated time points with 0.5 μg of either staphylococcal proteases aureolysin (AL) or staphopain A (SA; Biocentrum, Krakow, Poland). Sterile filtered bacterial supernatants (10–15 μL) from overnight cultures were incubated with recombinant MK protein for indicated time points before either SDS-PAGE followed by Western blot analysis or N-terminal sequencing. In a separate experiment, MK was incubated with COPD sputa or VAP aspirations (patient IDs: B6, A10, B9 and A2, A6, A7, respectively) for 1 and 18 h. Samples were then analyzed by SDS-PAGE and standard protein staining methods.

Zymography

Zymography was performed by using precast gels containing collagen (Bio-Rad). The material used in zymography assays were from COPD patients A1, A9 and B5 and VAP patients A3, A4 and B3, respectively.

N-Terminal Sequencing

The identification of MK fragments generated by *S. aureus* protease cleavage was performed by N-terminal se-

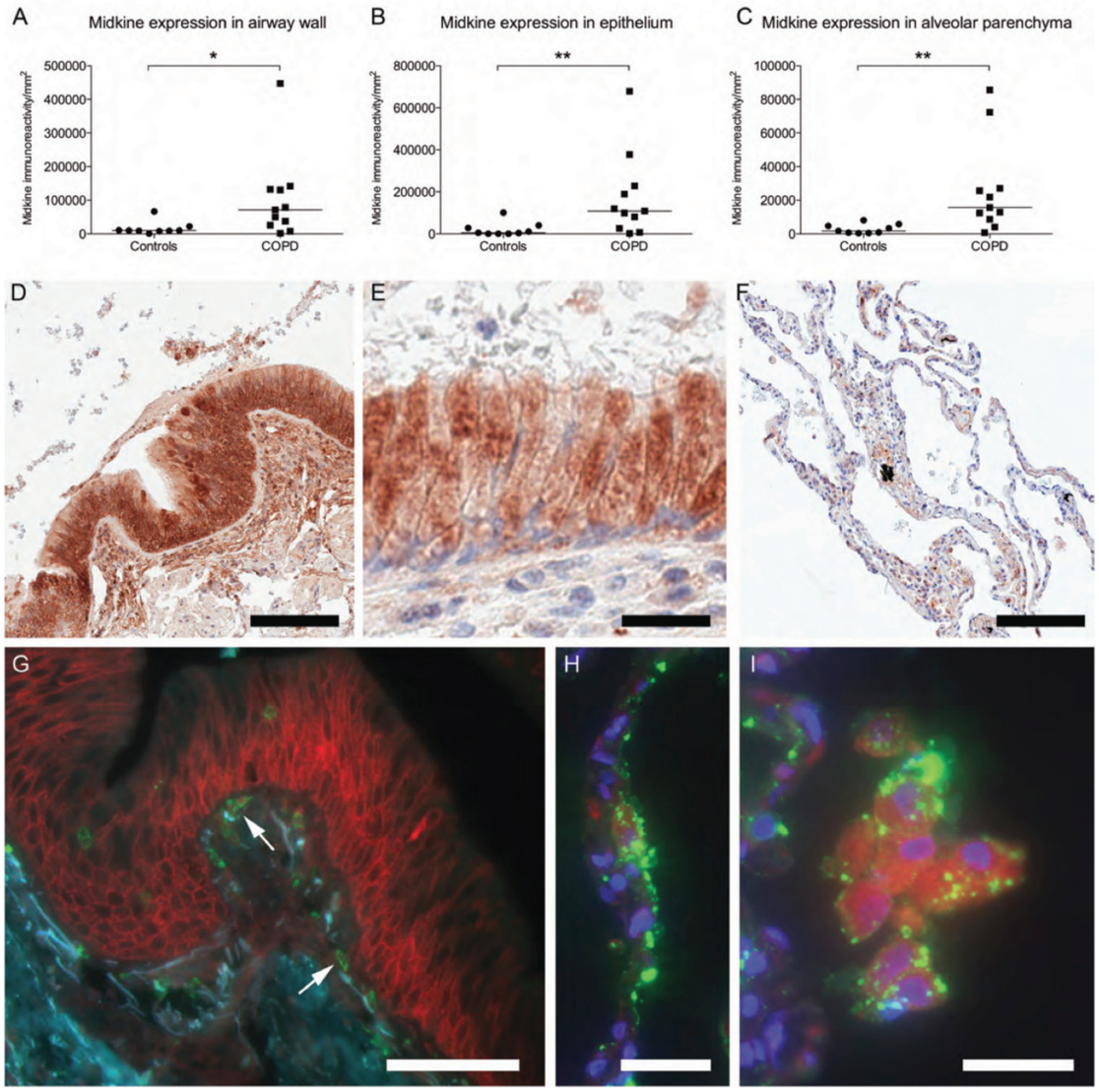

Figure 1. MK as detected by immunofluorescence in lung tissue from COPD. The immunoreactivity of MK expression, expressed as positive pixels per mm^2, in the small airway wall (A), small airway epithelium (B) and alveolar parenchyma (C). Data expressed as scatter dot plots and line indicate the median value. Asterisks show statistical difference when compared with controls: $*p < 0.05$, $**p < 0.01$. Immunohistochemical staining of MK in small airways (D), small airway epithelium at higher magnification (E) and alveolar parenchyma (F). Triple fluorescent immunohistochemical staining of nuclei (DAPI, blue) and MK (Alexa Fluor 555, red) together with MPO of neutrophils show weak colocalization (G) and surfactant protein A (SP-A) expressed by type 2 pneumocytes of the alveoli and is shown in green (H and I: Alexa Fluor 488). In (I), alveolar macrophages containing SP-A (that is, likely to have been phagocytosed) also show presence of MK. Scale bars: D, F = 200 μm; E, H, I = 50 μm; and G = 100 μm.

quencing by Edman degradation of eight sequential amino acids (HEJ Analyser; Karolinska Institutet, Stockholm, Sweden).

Molecular Modeling

Molecular modeling of MK was performed as described (4).

RESULTS

Expression of MK in Lung Tissue from COPD

Immunohistochemistry followed by morphometric image analysis showed that COPD airways, including the airway wall (Figure 1A), epithelial cells (Figure 1B) and the small airway parenchyma (Figure 1C) have a significantly higher expression of MK than the airways of controls. MK protein, as detected by immunohistochemistry in small airways (Figure 1D), as seen in a higher magnification (Figure 1E), is predominantly expressed in bronchial epithelial cells. In alveoli, weaker presence

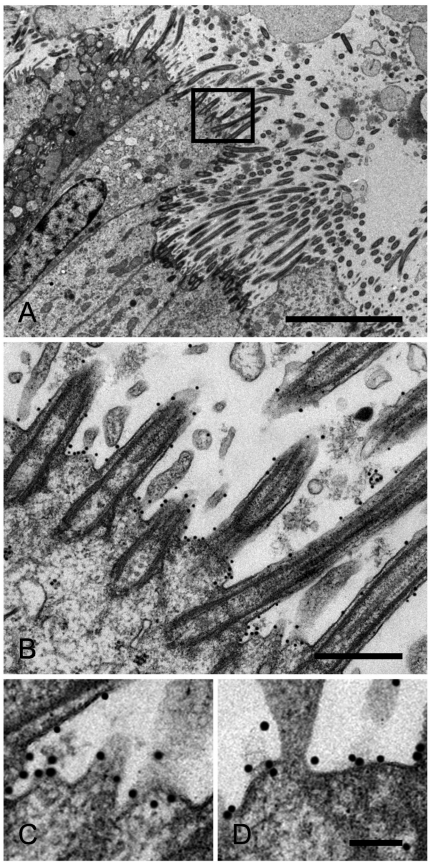

Figure 2. Ultrastructural detection of MK by immunoelectron microscopy of lung tissue in COPD. (A) Immunogold-labeling of ultrathin sections was performed on small airway lung tissue from a COPD patient. Colloidal gold particles indicate location of bound antibodies against MK. MK is present on the surface of cilia (B) and on the bronchial epithelial cell surface (C and D). Scale bars: A = 10 μm, B = 1 μm and C and D = 100 nm.

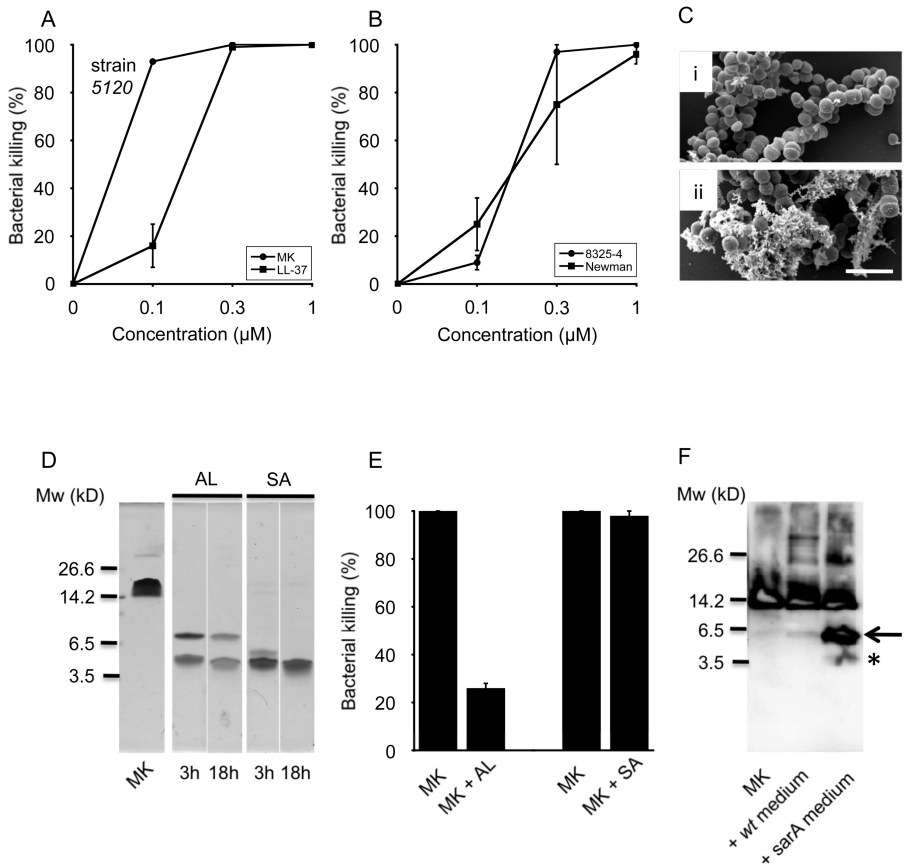

Figure 3. Bactericidal activity of MK against *S. aureus* is impaired by AL but not SA. (A) MK was compared with the classic HDP LL-37 in the viable counts assay using the *S. aureus* strain 5120 as target. Bacteria were incubated with increasing MK concentrations for 1 h and then plated, and CFUs were enumerated after an overnight incubation at 37°C. MK was the more potent antibacterial agent at lower concentrations, killing approximately 90% of the bacteria at 0.1 μmol/L. (B) MK antibacterial activity has potent bactericidal activity against the *S. aureus* strains Newman and 8325-4. (C) Scanning electron microscopy showing intact *S. aureus* after incubation in buffer alone for 1 h at 37°C (i) and disrupted bacteria with leakage of intracellular contents after incubation with MK (1 μmol/L) (ii). (D) MK was incubated with AL and SA for 3 and 18 h. Samples were separated by SDS-PAGE and the fragmentation pattern is visualized by Coomassie staining. AL generated bands of approximately 5 and 6 kDa, respectively, whereas SA generated two bands of similar sizes. (E) AL and SA degradation products of MK show differences in bactericidal activity. After incubation of MK with AL, only 25% killing was retained at a MK concentration corresponding to 1 μmol/L of the holoprotein. SA-generated fragments retained bactericidal activity in the order of the MK holoprotein. (F) Incubation of MK with culture supernatant from *S. aureus* 8325-4 *wt* and sarA (a strain overexpressing and releasing proteases) resulted in degradation of MK. A faint band is seen with supernatant from *wt*, and a distinct band is seen with sarA (arrow). In addition, a smaller band is observed in the latter case (*). The bands correspond to the molecular sizes generated with recombinant AL.

of MK was seen (Figure 1F). By immunofluorescent double staining, MK (red) was visualized in bronchial epithelium of small airways, showing weak colocalization with myeloperoxidase (green) of neutrophils. Neutrophils mainly showed submucosal positioning (Figure 1G). MK was also detected in the alveolar parenchyma, showing to some degree colocalization with SP-A of type 2 pneumocytes (Figure 1H). In addition, alveolar macrophages showed presence of both MK and SP-A (Figure 1I), capable of phagocytosing the latter (18).

To localize MK on an ultrastructural level, immunogold labeling followed by TEM analysis was performed (Figure 2). This process revealed the presence of MK on the apical surface and association with cilia of bronchial epithelial cells in COPD airways (Figures 2A, B); for closer detail see Figures 2C, D.

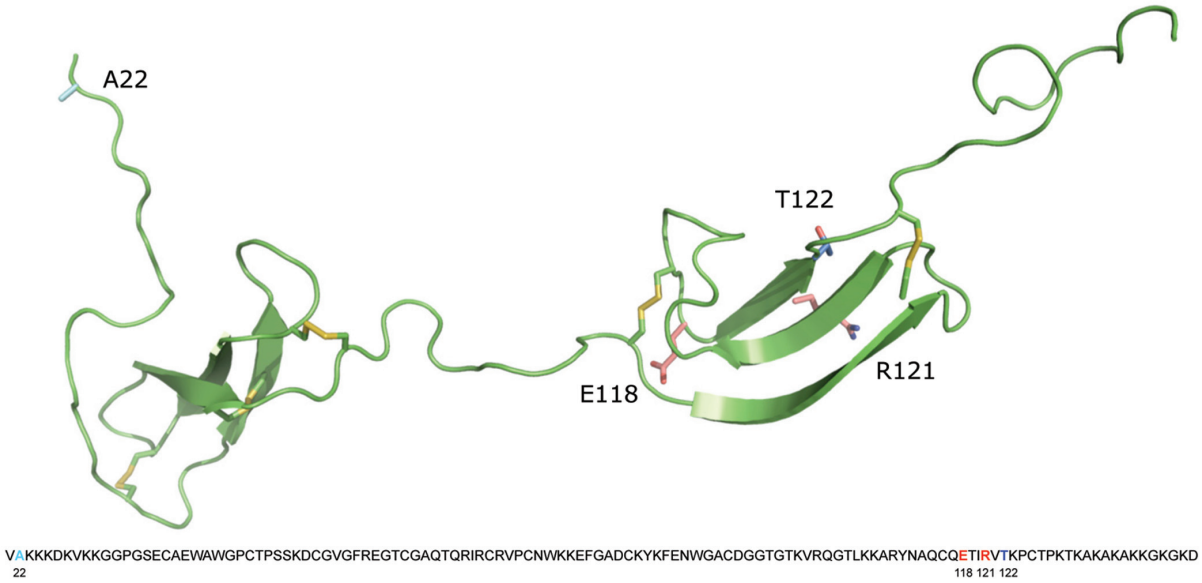

Figure 4. Molecular model of MK depicting the cleavage sites AL and SA, respectively. Fragments generated after cleavage with AL and SA, respectively, were characterized by N-terminal protein sequencing combined with mass spectrometry. The cleavage sites for AL are indicated in red and SA in blue. Both enzymes cleaved MK at A22.

Bactericidal Activity of MK Against Different Strains of *S. aureus*

A previous study identified MK as an antibacterial against *S. aureus* (4). In the current study, we demonstrate bacterial killing of *S. aureus* strains 5120, Newman and 8325-4 (Figures 3A, B). MK was the more potent antimicrobial agent, killing 5120 at lower concentrations than the classic HDP LL-37. Bacteria exposed to buffer alone remained intact, as imaged by standard error of the mean (Figure 3C, top panel i), whereas exposure to MK resulted in leakage of intracellular material from the bacteria, suggesting membrane disruption as a likely mode of action (Figure 3C, lower panel ii).

MK Fragments Generated by Staphylococcal Proteases

In the host–microbe encounter, bacterial proteases are likely to have access to constitutively expressed HDPs such as MK. Incubation of recombinant MK with the *S. aureus* proteases AL and SA for 3 h generated fragments, which remained present after 18 h of incubation (Figure 3D) as detected by protein staining. AL generated fragments of MK of the approximate size of 5 and 6 kDa, as estimated from the stained gel. N-terminal sequencing combined with mass spectrometry identified the band of higher molecular weight to correspond to the true NH terminus of the holoprotein, whereas the other band constituted a mixture of peptides located in the COOH terminus of the holoprotein. SA generated an MK fragment of the apparent size of 4 kDa, and N-terminal sequencing of this band showed a mixture of two equally abundant peptides—one corresponding to the true NH terminus of the holoprotein and one from the COOH terminus. These results are displayed in Figure 4.

To investigate the effect of AL and SA cleavage on MK bactericidal activity, MK was preincubated with respective protease and then used in the viable count assay. The results show that AL reduced MK killing to 25% (Figure 3E), whereas killing remained close to 100% after incubation with SA. MK was then incubated with supernatants from cultures of *S. aureus* wild-type (wt) strain 8325-4 or its protease overexpressing derivative sarA (Figure 3F). Immunoblotting revealed that MK remained intact after incubation with *wt* supernatant, whereas it was cleaved by the sarA supernatant. Taken together, these data demonstrate that MK is bactericidal toward *S. aureus* and that AL, but not SA, by proteolytic cleavage acts as a bacterial countermeasure to limit MK-mediated eradication.

COPD Sputa and Endotracheal Aspirations from VAP Patients Associated with *S. aureus* Infection Show Differences in MK Levels and Proteolytic Activity

Patient characteristics and the results of the virulence-associated genes screening are shown in Table 2. The average age of the patient groups was 73 years for COPD and 41 years for VAP. The VAP group spent an average of 3.3 d on a ventilator before the introduction of antibiotics or death occurred. None of the COPD patients required intubation. The results of the virulence gene screening revealed that only one bacterial isolate (A4) was positive for the *pvl* gene, and three (A4, B5, B9) were positive for the superantigen *tsst-1*. The *spa*-types t002 and t056 were found in two isolates each (A6, B7 and A7, A9, respectively). All tested strains were sensitive to methicillin.

Table 2. Characteristics of COPD and VAP patients, bacterial methicillin resistance and virulence-associated gene profile.

ID number	Gender	Age (years)	Underlying disease/injury	Days on ventilator	Methicillin resistance: cefoxitin	Virulence-associated genes		
						spa	*pvl*	*tsst-1*
A1	M	79	COPD	0	S	t091	N	N
A10	F	70	COPD	0	S	t9929	N	N
B1	M	42	COPD	0	S	t449	N	N
B5	F	79	COPD	0	S	t166	N	POS
B6	F	80	COPD	0	S	t094	N	N
B9	M	86	COPD	0	S	NT	N	POS
A2	F	67	Trauma	4	S	t005	N	N
A3	M	50	Cerebral hemorrhage	1	S	t2094	N	N
A4	M	25	Trauma	3	S	t127	POS	POS
A6	M	27	Trauma	3	S	t002	N	N
A7	M	46	Acute myocardial infarction	3	S	t056	N	N
A9	F	48	Subarachnoid hemorrhage	4	S	t056	N	N
B3	M	47	Cerebral infarction	3	S	t1743	N	N
B7	F	59	Neurosurgery	6	S	t002	N	N
B10	M	36	Trauma	3	S	t1931	N	N
C1	F	1	Sepsis	unknown	S	t065	N	N

The number of days on a ventilator was calculated from day of admittance until day of antibiotics administration or until death. S, sensitive; N, negative; POS, positive; NT, nontypeable.

Significantly less MK was detected in sputum from COPD patients (mean: 0.15 ng/mL, range 0–0.49; n = 5, 2 of 5 undetectable) than in endotracheal aspirations from VAP patients (n = 8, mean: 2.62 ng/mL, range 0–8.41; 1 of 8 undetectable) ($p = 0.030$ as determined by the Mann-Whitney *U* test; Figure 5A). Figure 5B shows degradation patterns of recombinant MK exposed for 1 or 18 h to either COPD sputa or VAP aspirations, respectively. In COPD sputum, MK degradation kinetics was more rapid than in aspirations from VAP patients. To elucidate why MK was degraded more rapidly in COPD sputa, zymography was performed. The substrate clearance area was quantified and shows that COPD sputa had a significantly higher proteolytic activity than VAP aspirations ($p = 0.01$, Figure 5C).

DISCUSSION

According to the World Health Organization, 65 million people are affected by COPD, and, based on data from 2005, the disease accounts for 5% of all deaths globally (http://www.who.int/respiratory/copd/burden/en). COPD exacerbations are linked to recurrent bacterial or viral infections that influence the outcome of the disease negatively. The most commonly isolated bacterial species are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, but *S. aureus* species are also found (19,20), particularly in elderly patients (21).

In healthy individuals, MK is constitutively expressed in bronchial epithelium of large airways and at least partially depends on retinoic acid (5). In the current study, we found COPD lung tissue to express MK in small airways, in particular within the airway wall, epithelium and parenchyma. This result is in contrast to healthy lungs, where MK is expressed in large but not small airways (5). Additionally, we found the presence of MK in type 2 pneumocytes and alveolar macrophages in the present study. One important question is whether MK concentrations reach bactericidal concentrations. In a previous study, using an air liquid system, we calculated the MK concentration of the airway surface liquid to an amount of 0.7 μmol/L (that is, a bactericidal concentration) (5). In addition, MK was detectable in induced sputum of healthy individuals (5). Several factors are likely to contribute to increased MK expression during *S. aureus* infection of the airways. The *MK* gene has a retinoic acid (RA)-responsive element in its promoter region, and several factors present during inflammation increase the generation of RA from vitamin A, for example, activation of TLR2 by peptidoglycan of gram-positive bacteria (e.g., *S. aureus)* (22,23). In addition, MK expression is enhanced by the proinflammatory transcription factor NF-κB, reactive oxygen species, tumor necrosis factor-α, and interleukin-1β (10,11). In severe infection, hypoxia of tissues may occur and, interestingly, hypoxia enhances MK expression via the transcription factor hypoxia-inducible factor 1-α (HIF-1α) (9).

Others found significantly elevated MK in sera from hypoxemic patients compared with healthy controls (24), but this study did not include examination of lung tissue or sputa. The difference in MK detection in lung tissue versus sputa questions the reliability of sputum as a sampling method. In addition, processing of sputa, for example by addition of the reducing agent dithiothreitol to lower viscosity, may result in dramatic differences with respect to detection limits of various readouts (25). The sputum and

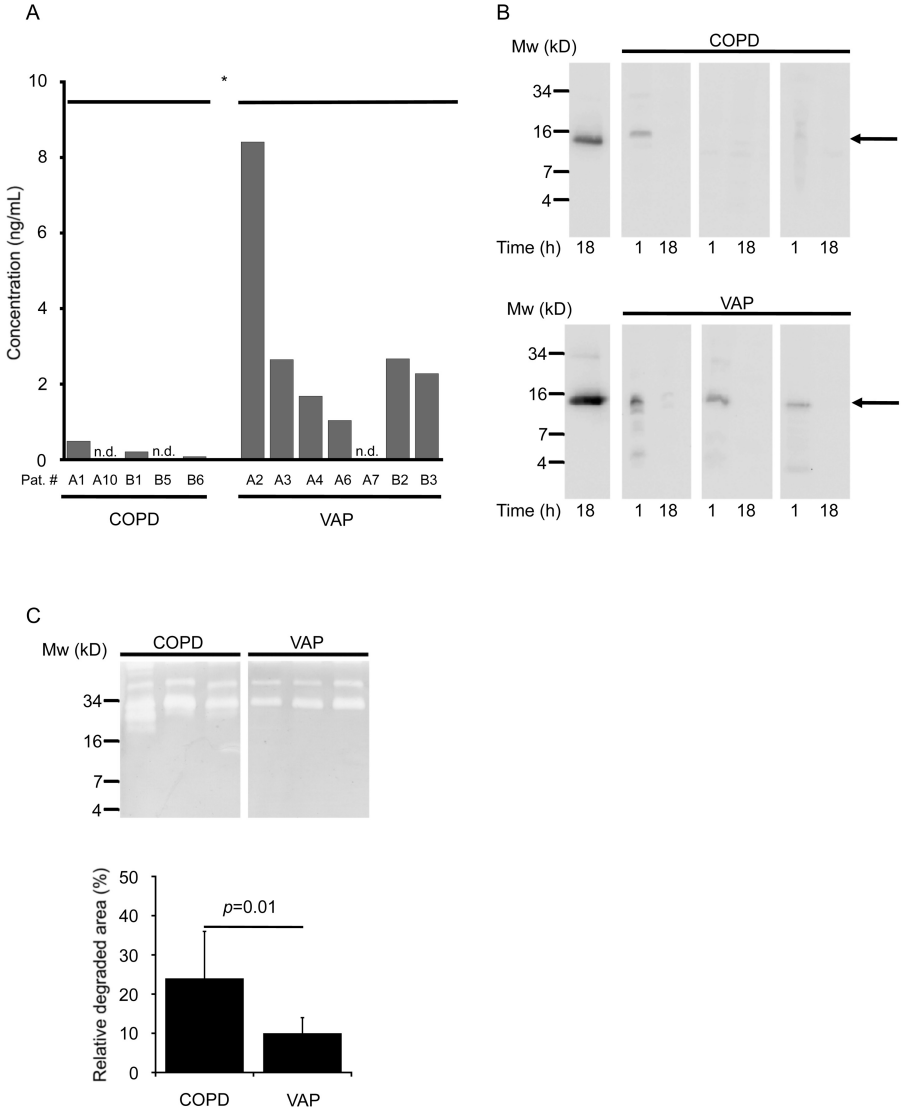

Figure 5. Detection and stability of MK in airway secretions from COPD and VAP, respectively. (A) Airway secretions from patients suffering from COPD exacerbations or VAP associated with *S. aureus* infection were analyzed for MK by ELISA. The results show significantly less MK in sputum from COPD patients compared with higher levels in VAP patients (p = 0.030, as determined by the Mann-Whitney U test). (B) To investigate the stability of MK in airway secretions, recombinant MK was incubated in sputa from three COPD patients or three VAP patients for 1 or 18 h and the integrity of the molecule was subsequently analyzed by Western blot. The kinetics of degradation was more rapid in COPD sputa, showing little or no MK remaining after 1 h in 2 of 3 patients. (C) To investigate and compare protease activity in airway secretions, sputa obtained from three COPD and three VAP patients were analyzed for protease activity by zymography. COPD sputa generated significantly larger clearing zones in the collagen matrix, indicating the presence of higher proteolytic activity ($p < 0.01$). The sizes of the clearing zones were measured, and the data represent mean ± SD. p value was calculated using the Student t test. n.d., Not detectable.

aspiration samples used in our study were without additions, increasing the reliability of the results.

Electron microscopy analysis located MK to the airway lumen and on the cilia of the epithelia of COPD airways (Figure 2). MK is believed to exert its antibacterial activity through bacterial membrane disruption and the extracellular location identified by electron microscopy, where it can encounter invading or colonizing pathogens, supporting the role of MK as an antibacterial factor (3). Earlier studies have localized the highest antibacterial activity of MK to the COOH-terminal half and identified its ability to kill gram-positive and gram-negative bacteria as well as fungi (3–5). *S. aureus* is named among the isolated pathogens found in acute exacerbations of COPD but, besides skin infections, it remains a more recognized cause of necrotizing pneumonia, HAP, VAP and upper airway infections. For these reasons, we used *S. aureus* as an experimental target for MK function and compared airway-expelled material from two different patient groups infected with this pathogen. As expected, MK readily killed all tested strains of *S. aureus in vitro*. AL but not SA destructed the antibacterial activity of MK. Both NH- and COOH-terminal regions of MK contain antibacterial activity, but only the activity of the COOH domain including the tail region equates to that of the holoprotein. Our data suggest that AL degrades the COOH-terminal half where its most potent antibacterial activity is found, although further experiments are needed to confirm this. Future studies will address what role, if any, the protease-generated fragments of MK have.

Not only do the proteases used in this study act on host proteins (26) such as MK, they also regulate the profile of the extracellular proteins on the bacterial surface (15). Thereby, these proteases have the ability to substantially influence bacterial virulence and host interplay. The theme is known from other host–pathogen encounters (27), and recent work has shown that proteases from *Streptococcus pyogenes* and *Finegoldia magna* act proteolytically to mediate bacterial evasion of host defense functions in skin infections (6). Bacterial colonization has over the last decade has become more recognized as a driving

force behind exacerbations in COPD. The role of bacterial proteases from pathogens more commonly associated with COPD exacerbations remains an open area of research.

S. aureus has many ways of evading the immune system, with expression of surface protein A, an adherence-promoting surface protein encoded by the *spa* gene, being one of them. The clinical isolates in our study showed genetic heterogeneity in terms of *spa* type; only two types were found in more than one patient. This result suggests a low risk of patient-to-patient or hospital-mediated spread. One of the strains in our study carried t127, which is also found in clones of US100. Methicillin resistance has historically been a larger problem in the U.S. than in Europe (14). Although staphylococcal virulence does not depend on methicillin resistance alone but relies on a combination of factors, it creates major problems for hospitals and health care professionals. Frequency of MRSA isolation remains low in Sweden (http://www.smittskyddsinstitutet.se [continuously updated information]), and fortunately, we found all strains in this study sensitive to methicillin.

That we detected MK in COPD airways by histology analysis but not in sputa led us to investigate whether COPD sputa contained more proteolytic activity than VAP aspirations. Degranulation of extravasated neutrophils in the airways together with a protease–protease inhibitor imbalance is a hallmark of exacerbations in COPD as described in the article by Celli and Barnes (28). That COPD samples showed larger clearing zones in the zymography assay is likely due to higher concentrations of proteases originating from the increased number of neutrophils, which is a hallmark of the condition. The serine proteases not only degrade MK, as shown in this study, but also contribute to epithelium injury, increased mucus production and chemokine production (29). Because both patient groups were infected with *S. aureus*, it appears that the contribution of host proteases to MK degradation in COPD is greater than the contribution by bacterial proteases. The patient groups differ by age and ventilation therapy and the sample size is small. Also, the VAP group may not suffer from the protease inhibitor imbalance or other unknown COPD-related innate immunity deficits. The composition of sputum (COPD) compared with tracheal aspirates (VAP) could differ. One example is the levels of anionic molecules (for example, mucins, free DNA and osteopontin) that could interact with MK and thus affect measurements. In addition, we cannot rule out that contaminating saliva could have a diluting effect on the MK concentrations observed in COPD. However, in a previous study, sputum was induced through inhalation of nebulized sodium chloride in healthy individuals. In these samples, MK was detectable using the same ELISA as used in the current study (5).

CONCLUSION

Our data demonstrate the possibility that *S. aureus* modulates and corrupts host airway defense lines such as MK by fragmentation in both immuno-competent and -suppressed patient groups.

ACKNOWLEDGMENTS

We thank Pia Andersson and Maria Baumgarten for excellent technical assistance and Staffan Arvidson for providing the *S. aureus* strains 8325-4 and sarA.

This work was supported by the Swedish Research Council (projects A0615601 HML and 2010-4224AE); the Swedish Heart and Lung Foundation (20100164); the Medical Faculty of Lund University; Swedish Government Funds for Clinical Research (ALF); and the foundations of Bergh, Greta and Johan Kock, and Alfred Österlund.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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