Elevated levels of macrophage-stimulating protein in induced sputum of patients with bronchiectasis

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We recently showed that macrophage-stimulating protein (MSP), a serum protein homologous to hepatocyte growth factor, promotes ciliary motility by activating its receptor, RON, on the airway ciliated epithelium. To investigate the functional involvement of MSP and RON in bronchiectasis, in which mucociliary clearance (MCC) is impaired, first we examined RON expression on the bronchial ciliated epithelium of patients with bronchiectasis. We confirmed RON expression at the apical surface of bronchial ciliated epithelium of patients with bronchiectasis as well as those of normal human bronchus. Next, we examined whether MSP is present in sputum of patients with bronchiectasis and normal control subjects. By Western blotting, we found that half of the MSP in sputum is present as a biologically active α/β chain heterodimer (mature MSP). In addition, we found that the MSP concentrations in sputum were significantly elevated in patients with bronchiectasis (n = 8; 16±8 ± 3±0 ng ml⁻¹) compared with normal controls (n = 9; 8±4 ± 2±4 ng ml⁻¹; P < 0±05). In contrast, the difference in concentrations of serum MSP (pro-MSP) was not significant between the two groups. These results indicate that (i) MSP is supplied to the airways and converted to a biologically active form, (ii) MSP is increased in patients with bronchiectasis compared with normal controls. Taken together, our findings suggest that increased MSP may be involved in compensation for impaired MCC in bronchiectasis.

Key words: macrophage-stimulating protein; RON; bronchiectasis; induced sputum; mucociliary clearance.

Introduction

Bronchiectasis is a disease of mucus hypersecretion and recurrent airway inflammation that causes destruction of the ciliated epithelium and submucosa with elastic and muscular tissue degeneration (1,2). It has been demonstrated that mucociliary clearance (MCC) is impaired in the airways of patients with bronchiectasis (3,4). Impaired MCC may be due to abnormal mucociliary function, abnormal load and rheology of mucus, or abnormal interaction between cilia and mucus (5). The lungs of patients with bronchiectasis are frequently colonized by Haemophilus influenzae and Pseudomonas aeruginosa (6). Soluble products Pseudomonas aeruginosa, such as pyocyanin and l-hydroxyphenazine, were found to inhibit ciliary beat frequency (CBF) of human respiratory cilia in vitro and cause the reduction of tracheal mucus velocity in vivo (7,8). Thus, ciliary dysfunction may be involved in the impairment of MCC in patients with bronchiectasis.

Recently, we have demonstrated that the receptor tyrosine kinase RON is expressed on the airway ciliated epithelium, and that its ligand macrophage-stimulating protein (MSP) promotes ciliary motility in these cells by activating RON (9). These findings suggest that MSP functions as one regulatory factor of ciliary motility and plays an important role in the airway mucociliary transport. To date, however, the biological significance of MSP in the airway mucociliary transport in vivo has not been clarified.

MSP is an 80-kD serum protein that was originally found to stimulate murine resident peritoneal macrophages to make a chemotactic response to C5a (10). MSP belongs to a family characterized by the presence of a highly conserved triple disulphide loop structure (kringle domain). The family includes prothrombin, plasminogen, urokinase and hepatocyte growth factor, the latter of which shows high homology to MSP (45% identity at the amino acid level) (11–14). Proteins in this family are secreted as single-chain precursors that acquire biological activity upon cleavage by specific serine proteases. It has been shown that MSP is synthesized by hepatocytes (11,13,15) and secreted as biologically inactive pro-MSP, the single-chain precursor of MSP, into the circulation. In addition, pro-MSP is
reported to be cleaved to a disulphide-linked α/β hetero-
dimer (mature MSP) by serine proteases such as enzymes of
the intrinsic coagulation cascade (16).

To explore the role of MSP and RON in bronchiectasis,
initially we investigated whether RON is expressed on the
bronchial ciliated epithelium of patients with bronchiet-
asis. In addition, we investigated whether MSP is present in
induced sputum and measured the concentrations of MSP
in induced sputum of patients with bronchiectasis and
normal control subjects. In this paper, we show that RON
is expressed at the apical surface bronchial ciliated
epithelium of patients with bronchiectasis. Furthermore,
we show that half of the MSP in sputum is present as
biologically active mature MSP, and that MSP concentra-
tions in sputum are significantly increased in patients
with bronchiectasis compared with normal controls.

Methods

SUBJECTS

Seventeen subjects participated in the study after giving
informed consent. Eight were patients with bronchiectasis.
Nine were control subjects who had no history of
pulmonary diseases. The chest roentgenograms and pul-
monary function tests of nine controls were normal. The
study was approved by the Institutional Review Board of
Kumamoto University. Patients with bronchiectasis were
diagnosed by chest roentgenogram and computed tomo-
graphy. All cases of bronchiectasis occurred with post-
infectious causes and recurrent persistent infections. None
of them had immunoglobulin deficiency or Kartagener’s
syndrome. All subjects underwent clinical, hematological,
and chemical examinations in order to exclude acute
infectious exacerbations. Forced vital capacity (FVC) and
forced expiratory volume in 1 sec (FEV1) were measured by
a dry rolling seal spirometer (Chestac-65V, Chest Co.,
Tokyo, Japan). The clinical characteristics of these subjects
are shown in Table 1.

SPUTUM INDUCTION AND PROCESSING

Before collection of samples subjects were asked to rinse
their mouth, swallow water, and blow their nose to
minimize contamination with saliva and post-nasal drip.
Then inhalation of hypertonic saline (3%) was performed
using an ultrasonic nebulizer (NE-U11, Omron Co., Tokyo,
Japan). Inhalation of hypertonic saline was continued for
15–20 min until the sputum volume was over 1 ml. The
nebulizer generated particles with a mass diameter of 1–5 μm
and had an output of 1·5 ml min⁻¹. Subjects were
encouraged to cough throughout the procedure, and they
regularly interrupted inhalation of hypertonic saline in
order to expectorate sputum into a clean plastic dish.

The assessment of the quality of the obtained samples
was performed as described by Pin et al. (17). One thousand
microlitres of sputum was immediately cooled on ice and
combined with 950 μl of phosphate buffered saline (PBS)
and 50 μl of 2 mM p-amidinophenyl methanesulphonyl
fluoride hydrochloride (APMSF) (Wako Pure Chemical,
Osaka, Japan). The samples were then homogenized and
centrifuged at 14 000 rpm at 4°C for 20 min. The obtained
supernatants were frozen immediately at −70°C.

IMMUNOHISTOCHEMISTRY

Subsegmental bronchi (diameter: 3–6 mm) of normal
control and patients with bronchiectasis were obtained
from surgical and autopsy samples. Immediately after the
tissues were obtained, they were mounted in OCT 4583
embedding compound (Miles Inc, Elkhart, IN, U.S.A.) and
then frozen in liquid nitrogen. Serial cryostat sections (6-μm
thick) cut in a MICROM cryomicrotome were air dried,
and fixed in acetone for 10 min at 4°C. After washing with
PBS three times, cryostat sections were treated with 5 mM
orthoperiodic acid solution for 10 min to block endogenous
peroxidase activity as described (18). The sections were
stained with a rabbit polyclonal antibody against RON
(0·2–0·5 μg ml⁻¹) (Santa Cruz Biotechnology, Inc., CA,
U.S.A.) by the indirect immunoperoxidase method using
horseradish peroxidase (HRP)-conjugated anti-rabbit IgG
(Amersham International, Little Chalfont, U.K.). Perox-
idase activity was visualized using 3,3-diaminobenzidine
(Dojindo, Kumamoto, Japan) as a substrate. To confirm
the specific reactivity of anti-RON with the RON protein,
one of the serial sections was incubated with a mixture of
anti-RON antibody and an excess molar ratio of antigen
peptide (Santa Cruz Biotechnology, Inc.), then processed as
described above.

IMMUNOPRECIPITATION AND WESTERN
BLOTTING

Each 300 μl sample of induced sputum obtained from
bronchiectasis subjects and from control subjects was
incubated for 6 h at 4°C with protein G-Sepharose
(Pharmacia, Uppsala, Sweden) precoated with a mouse
monoclonal anti-human MSP antibody (MSP89-2S, Amer-
ican Type Culture Collection HB-10522, Rockville, MD)
with an equal volume of 2× Triton lysis buffer [100 mM
Hepes, pH 7·4, 2% Triton X-100, 300 mM NaCl, 2 mM
EDTA and protease inhibitors (50 μg ml⁻¹ aprotinin, 1 mM
PMSF, 100 μM leupeptin, 25 μM pepstatin A)]. Immune
complexes were collected on protein G-Sepharose. Immu-
noprecipitates were boiled for 3 min at 100°C in sodium
dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-
PAGE) sample buffer with 5% 2-mercaptoethanol (2-ME),
then resolved by SDS-PAGE using 10% polyacrylamide as
described by Laemmli (19). Recombinant human pro-MSP
and MSP (Toyobo Co., Shiga, Japan) were also applied to
the gel for reference markers. After electrophoresis,
proteins were transferred onto a PVDF membrane (Nihon
Millipore Ltd, Yonezawa, Japan). The α-chain of mature
MSP and pro-MSP were evaluated with a rabbit polyclonal
anti-human MSP antibody (Toyobo Co.). Specific binding
was detected using the enhanced chemiluminescence (ECL)
detection system (Amersham International).
QUANTIFICATION OF MSP IN SERUM AND INDUCED SPUTUM

MSP concentrations in serum and the supernatant of induced sputum were measured by sandwich-type ELISA. This ELISA system detects both MSP and pro-MSP. Briefly, a microtitre plate was coated overnight at 4°C with a mouse monoclonal anti-human MSP antibody. After blocking with 3% bovine serum albumin in PBS, the plate was incubated with diluted standards or samples. After washing, the plate was incubated with a rabbit polyclonal anti-human MSP antibody, followed by HRP-conjugated anti-rabbit IgG (Amersham International). Specific binding was detected by incubating the plate with 0.05 M phosphate-citrate buffer containing 0.4 mg ml⁻¹ o-phenylenediamine dihydrochloride and 0.4 mg ml⁻¹ urea hydrogen peroxide (Sigma Chemical Co., St. Louis, MO, U.S.A.). The absorbance at 450 nm was measured in an automated microplate reader (Molecular Devices Corp., Sunnyvale, CA, U.S.A.). MSP concentrations were determined by interpolation of their absorbance from a standard curve.

STATISTICAL ANALYSIS

Results are shown as mean ± SEM. The differences between groups were compared with Mann–Whitney’s U-tests. A value of P<0.05 was considered significant.

Results

We studied eight patients with bronchiectasis and nine control subjects. There was no significant difference in FVC (%pred) and FEV₁/FVC (%) between the two groups. 

TABLE 1. Characteristics of study population

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Smoking (pack-years)</th>
<th>FVC (%pred)</th>
<th>FEV₁/FVC (%)</th>
<th>Bacteria in sputum</th>
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<tbody>
<tr>
<td>Patients with bronchiectasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>47</td>
<td>0</td>
<td>70-8</td>
<td>94-8</td>
<td>Normal flora</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>67</td>
<td>0</td>
<td>82-6</td>
<td>77-6</td>
<td><em>H. influenzae</em></td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>57</td>
<td>25</td>
<td>92-2</td>
<td>89-3</td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>46</td>
<td>50</td>
<td>61-2</td>
<td>52-7</td>
<td>Normal flora</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>66</td>
<td>0</td>
<td>72-1</td>
<td>79-0</td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>62</td>
<td>0</td>
<td>79-0</td>
<td>75-0</td>
<td>Normal flora</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>63</td>
<td>0</td>
<td>134-0</td>
<td>89-1</td>
<td>Normal flora</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>71</td>
<td>0</td>
<td>127-7</td>
<td>58-6</td>
<td><em>H. influenzae</em></td>
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<tr>
<td>Mean</td>
<td></td>
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<td>90-0</td>
<td>77-0</td>
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<tr>
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<td>6-4</td>
<td>5-3</td>
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<td>Normal controls</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>51</td>
<td>0</td>
<td>110-0</td>
<td>85-0</td>
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<tr>
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<td>Normal flora</td>
</tr>
<tr>
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<tr>
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<tr>
<td>5</td>
<td>M</td>
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<td>40</td>
<td>96-0</td>
<td>77-0</td>
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<tr>
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<td>F</td>
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<td>0</td>
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<td>84-8</td>
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<tr>
<td>7</td>
<td>M</td>
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<td>0</td>
<td>102-0</td>
<td>88-0</td>
<td>Normal flora</td>
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<tr>
<td>8</td>
<td>M</td>
<td>33</td>
<td>0</td>
<td>110-1</td>
<td>108-2</td>
<td>Normal flora</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>30</td>
<td>0</td>
<td>116-1</td>
<td>96-1</td>
<td>Normal flora</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>106-9</td>
<td>86-8</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td></td>
<td></td>
<td>6-4</td>
<td>3-1</td>
<td></td>
</tr>
</tbody>
</table>

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control subjects with a monoclonal anti-human MSP antibody. Western blotting with a rabbit polyclonal anti-human MSP antibody that detects mature MSP and pro-MSP showed that these sputa contain comparable levels of MSP α-chain and pro-MSP (Fig. 2). These results indicate that half of the MSP in sputum is present as biologically active mature MSP in both patients with bronchiectasis and normal controls.
Next, to investigate the role of MSP in bronchiectasis, we measured concentrations of MSP in serum and induced sputum of patients with bronchiectasis and normal control subjects. As shown in Table 2, serum MSP (pro-MSP) levels were 7.8 ± 0.5 μg ml⁻¹ in patients with bronchiectasis and 8.1 ± 0.3 μg ml⁻¹ in normal controls. There was no significant difference between the two groups. On the other hand, MSP levels in induced sputum were 16.8 ± 3.0 ng ml⁻¹ in patients with bronchiectasis and 8.4 ± 2.4 ng ml⁻¹ in normal controls (P < 0.05). Thus, MSP levels were significantly increased in sputum but not in the serum of patients with bronchiectasis as compared with normal controls.

**Discussion**

It has been recognized that the effectiveness of MCC depends on three factors: ciliary function, secretion of mucins and other macromolecules, and fluid secretion. In particular, ciliary function and interaction between cilia and mucus have been shown to be critical for airway mucociliary transport (5). In our recent study, we showed and mucus have been shown to be critical for airway mucociliary transport (5). In this study, we confirmed that RON is also expressed at the apical surface of bronchial ciliated epithelium of patients with bronchiectasis as well as those of normal human bronchus. In addition, we confirmed that MSP is present in the sputum of patients with bronchiectasis and normal control subjects, and that half of the MSP in sputum is present as a biologically active α/β chain heterodimer (mature MSP). Previous studies have shown that MSP is produced by hepatocytes (11,13,15) and secreted into the circulation as biologically inactive pro-MSP, the single-chain precursor of MSP, and maintained in serum as pro-MSP (20). Furthermore, pro-MSP has been shown to be cleaved to a biologically active α/β chain heterodimer by serum kallikrein and coagulation Factors XIIa and X in vitro (16). Our results suggest that MSP is supplied to the airways from the circulation and converted to an active form. Taken together with the finding that the MSP receptor, RON, is localized to the apical surface of airway ciliated epithelium, it seems reasonable to conclude that MSP in sputum binds to and activates RON on the airway ciliated epithelium resulting in stimulation of ciliary motility in vivo.

Wang et al. previously showed that resident murine peritoneal macrophages, one of the target cells for MSP, have enzymatic activities that proteolytically cleave pro-MSP (20). These results suggest that control of MSP activity can occur at the level of the target cell by proteolytic cleavage of pro-MSP to mature MSP. Since the mechanism of proteolytic cleavage of serum pro-MSP to mature MSP in sputum remains to be clarified, it will be of interest to explore whether airway ciliated epithelial cells, one of the target cells for MSP, contain enzymatic activity that proteolytically cleaves pro-MSP to mature MSP.

The *Haemophilus influenzae* and *Pseudomonas aeruginosa* frequently colonize in the respiratory tract of patients with bronchiectasis (6), and these bacterial and inflammatory cell products have been shown to have a detrimental effect on the mucociliary transport apparatus. For example, soluble products of *Haemophilus influenzae* and *Pseudomonas aeruginosa* can decrease CBF (21,22); pyocyanin, 1-hydroxyphenzamine and rhamnolipids were found to be the putative agents in *Pseudomonas aeruginosa* (7,23,24). Furthermore, the following inflammatory mediators released into the airway lumen during inflammation have been shown to decrease CBF: hydrogen peroxide (25), human neutrophil elastase (26) and neutral protease (27). Thus, the effect of these bacterial products and inflammatory mediators in vivo is cilioinhibition, and this inhibition

**TABLE 2. MSP concentrations in serum and induced sputum**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number</th>
<th>Serum (μg ml⁻¹)</th>
<th>Induced sputum (ng ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchiectasis</td>
<td>8</td>
<td>7.8 ± 0.5</td>
<td>16.8 ± 3.0*</td>
</tr>
<tr>
<td>Normal controls</td>
<td>9</td>
<td>8.1 ± 0.3</td>
<td>8.4 ± 2.4</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM.
*P < 0.05 compared with normal controls.
References


Acknowledgements

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Elevated Levels of MSP in Sputum of Bronchiectasis


