Elevated levels of high mobility group box chromosomal protein-1 (HMGB-1) in sera from patients with severe bacterial pneumonia co-infected with influenza virus

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ABSTRACT

Plasma levels of high mobility group box chromosomal protein-1 (HMGB-1), as well as of other inflammatory molecules such as interleukin-6 (IL-6), regulated on activation normal T-cell expressed and secreted (RANTES), and soluble intercellular adhesion molecule-1 (sICAM-1), were determined in patients with bacterial pneumonia co-infected with influenza virus. HMGB-1 levels were significantly elevated in these patients, as compared to patients undergoing mild bacterial pneumonia alone ($p<0.01$). Among cases of co-infection, we found a significant correlation between the concentration of HMGB-1 and white blood cell counts ($p<0.05$, $r=0.612$). Levels of IL-6 were also higher in these patients than in patients with bacterial pneumonia alone ($p<0.05$), despite similar levels of RANTES and sICAM-1 in the two groups. These data suggest that HMGB-1 is involved in the pathogenesis of severe bacterial pneumonia co-infected with influenza virus.

Key Words:

HMGB-1, influenza virus, bacterial pneumonia, co-infection

Abbreviations:

HMGB-1, high mobility group box chromosomal protein-1; ELISA, enzyme-linked immunosorbent assay; *S. pneumoniae*, *Streptococcus pneumoniae*; IL-6: interleukin-6, TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; COX, cyclooxygenase; PGE2, prostaglandin E2; *P. aeruginosa*, *Pseudomonas aeruginosa*; MPO, myeloperoxidase; RANTES, regulated on activation normal T-cell expressed and secreted; sICAM-1, soluble intercellular adhesion molecule-1; BALF, bronchoalveolar lavage fluid

INTRODUCTION

Influenza virus infection is a major respiratory infectious disease that generally induces bronchitis, although it can occasionally lead to fatal pneumonia in the elderly when complications involve bacterial infections [1]. Secondary/mixed bacterial pneumonia has been reported as a severe complication related to influenza virus infection, suggesting its etiological importance in the morbidity and mortality of elderly patients [1, 2].

We have already reported the case of a patient with severe bacterial pneumonia co-infected with influenza virus [3] and demonstrated the increased severity of bacterial pneumonia due to influenza virus co-infection in patients [4]. Moreover, we have previously shown the fulminant pathological changes observed in the lungs of mice inoculated with *S pneumoniae* 2 days after influenza virus co-infection, including the kinetics of viral titers, bacterial numbers, and immune responses such as the release of cytokines and chemokines. We showed that certain critical immune mediators, such as Toll-like receptor (TLR)/mitogen-activated protein kinase (MAPK) signaling, cyclooxygenase (COX) expression, and prostaglandin E2 (PGE2) production, were increased [5]. Furthermore, in mice, chronic *P. aeruginosa* infection is exacerbated by influenza virus, and decreased

neutrophil function due to viral infection may induce fulminant pneumococcal pneumonia in such mice [6-8]. These data suggested that synergistic effects of co-infection with influenza virus and bacteria mediated through immune reactions were involved in the pathogenesis of severe pneumonia.

High mobility group box chromosomal protein-1 (HMGB-1) has been reported as a critical inflammatory marker with an important etiological role in severe infectious diseases, especially in sepsis, but has not been studied in severe pneumonia related to influenza [9, 10]. We measured the levels of HMGB-1 and other immunological mediators, including interleukin-6 (IL-6), regulated on activation normal T-cell expressed and secreted (RANTES), and soluble intercellular adhesion molecule-1 (sICAM-1), in patients diagnosed with bacterial pneumonia co-infected with influenza virus and compared the levels with white blood cell (WBC) counts and C-reactive protein (CRP) levels, used as common markers of pneumonia severity.

MATERIALS and METHODS

Patients and diagnostic criteria

Twelve consecutive patients who attended our hospital between December 2004 and April 2005 were given a diagnosis of concurrent influenza virus infection and bacterial pneumonia on admission. Influenza virus infection was confirmed in nasopharyngeal swab samples using a rapid antigen detection kit (Espline Influenza A&B-N; Fujirebio, Tokyo, Japan). We performed cell culture and/or RT-PCR (Influenza Virus Primer Set kit; Maxim Biotech Inc., San Francisco, CA, USA) from patients` sputum and confirmed whether the influenza virus was present. We also made clinico-epidemiological suppositions.

These patients also had fever, cough, and yellow sputum and their chest X-rays revealed pulmonary infiltration. Specific bacteria, including *S. pneumoniae*, *H. influenzae*, *S milleri*, *S. aureus*, and *M. catarrhalis* were isolated from their sputum. Patients # 2, 7, 8, 10, 11, and 12 (Table 1) were hospitalized and their blood samples were collected at admission. Other patients were treated as outpatients and their blood samples were collected at the first visit.

Patients were given a diagnosis of bacterial pneumonia alone (n=18) when nasopharyngeal swabs were negative for influenza virus antigen and/or cell culture and RT-PCR from sputum were negative for influenza virus, even though they had a cough and yellow sputum accompanied by infiltration shadows on the chest X-ray.

Pneumonia was diagnosed as the presence of symptoms of lower respiratory tract infection along with a new infiltrate on the chest X-ray and no emerging alternative diagnosis [11]. Chest X-ray findings were reviewed and blindly assessed by three physicians who then determined the level of infiltration.

All patients provided informed consent to participate in the study and the study protocol was approved by the ethics committee of Nagasaki University.

Measurement of immune mediators

Serum concentrations of IL-6, RANTES, and sICAM-1 were measured using enzyme-linked immunosorbent assay kits (Quantikine; R&D Systems, Minneapolis, MN). Serum samples were diluted 1: 10 for RANTES and sICAM-1 before each assay, except for IL-6, which was measured in undiluted samples. HMGB-1 was also examined by ELISA (Shino-test; Sagamihara, Kanagawa, Japan) as previously described [12, 13].

Data collection and statistical analysis

Clinical data were statistically analyzed with the Mann-Whitney U-rank test, a nonparametric statistical test. Where necessary, the results were further corrected using Bonferroni's method. Spearman's rank correlation was used to examine the relationship among various parameters. All data are expressed as the mean value ± S.D. and were analyzed using StatView® software (Abacus Concepts, Cary, NC, USA). A p -value below 0.05 was considered to be a statistically significant.

RESULTS

Patient characteristics

Table 1 shows the baseline characteristics of the 12 patients (5 male and 7 female) with both bacterial pneumonia and influenza. Their mean age (51.0±21.9 years) did not differ substantially from that of the group with bacterial pneumonia alone (58.3±23.3 years). *H. influenzae* was isolated from six of the 12 patients, *S. pneumoniae* from three, *S. aureus* from two, and *P. aeruginosa* was isolated from one patient. In the group of patients with bacterial pneumonia alone, *S. pneumoniae* was isolated from 6 patients, *H. influenzae* from 7, and *M. catarrhalis* from 5 patients (data not shown).

Only co-infected patients showed complications, including chronic lung disease [tuberculosis (n=4), COPD (n=3)], chronic heart failure (n=2), and kidney stones (n=1). In contrast to co-infected patients, only 3 of 18 patients with bacterial pneumonia without influenza had chronic lung disease ($p<0.05$, data not shown).

Fever was the most common symptom in co-infected patients; however, temperature was at a similar level (37.6±0.8°C vs. 37.1±0.4°C) in the two groups. Heart rate (73.1±13.7/min vs. 67.3±17.9/min) and respiratory rate (20.5±3.0/min vs 18.7±6.1/min) were

also similar. In chest X-rays, severe infiltrations (>2/3 in both lung fields) were found in Patient #7 and moderate shadows were found in Patients #2 and #10. Other patients had mild infiltration shadows on chest X-rays (data not shown).

Compared with levels in patients with bacterial pneumonia alone, co-infected patients had increased levels of WBC (8252.5±2308.6/mm^3 vs. 6733.3±1752.9/mm^3; $p<0.05$) and CRP (4.6±5.1mg/L vs. 2.7±6.1 mg/L; $p<0.05$).

Concentrations of IL-6, RANTES, s ICAM-1, and HMGB-1 in serum

Compared with levels in patients with bacterial pneumonia alone, co-infected patients had significantly higher levels of IL-6 (30.9±11.3 pg/ml vs. 59.4±46.4 pg/ml; $p<0.05$, Table 2). RANTES and sICAM-1 levels did not differ significantly between the two groups. However, HMGB-1 was significantly higher in sera from co-infected patients than in the sera from patients with bacterial pneumonia alone (45.7±17.0 ng/ml vs. 14.8±4.3 ng/ml; $p<0.01$, Fig. 1A).

Correlation between HMGB-1 and WBC count

In co-infected patients, we examined the relationships between the serum concentration of HMGB-1 and the inflammatory markers WBC, CRP, and IL-6. There was no significant correlation between CRP or IL-6 and the levels of HMGB-1. However, a significant correlation was found between the WBC count and the concentration of HMGB-1 ($r=0.612$; $p<0.05$, Fig. 1B).

DISCUSSION

The “Spanish flu” pandemic of 1918 was responsible for more than 20 million deaths worldwide, a new strain of influenza (H5N1) caused many deaths due to severe lung damage in Hong Kong in 1997, and recently, an avian-type influenza has become epidemic[15,16]. However, the pathogenesis of lung damage remains controversial. Several studies, including one from our group, have identified acute lung haemorrhage with massive pneumonia in the lungs of mice co-infected with influenza virus and bacteria, whereas infection with either influenza virus or *S. pneumoniae* alone induced only moderate pneumonia [5, 17]. Co-infected mice died significantly earlier than mice infected with one type of organism, due to severe fulminant lung damage rather than septic changes. The mechanisms by which the patients’ conditions become more severe as a result of synergy between the influenza virus and bacteria are mediated through hyperimmune reactions. Bacterial numbers, but not viral titres, were increased [5]; however, viral infection may be more critical because former viral infection injures lung epithelial cells and accelerates the latter bacterial proliferation. In addition, it was reported that antiviral drugs are more effective than antibiotics for preventing secondary bacterial pneumonia after influenza virus infection in mice [17].

In this study, we analyzed several immune mediator molecules in sera from patients with bacterial pneumonia and influenza and compared them with levels found in sera from patients with bacterial pneumonia alone. Clinical findings and laboratory data of the patients in this study were not especially high, perhaps due to the characteristics of influenza type B, which is more mild than type A [1, 2] and was dominant in that season epidemically. However, we found significant differences in WBC counts and CRP levels (normal range <0.3 mg/dL) between the groups.

Levels of HMGB-1 and IL-6, especially HMGB-1, were significantly higher in patients with bacterial pneumonia co-infected with influenza virus. HMGB-1 might be involved in the pathogenesis of co-infection. HMGB-1 has recently been reported to be an important late mediator of endotoxic shock, intra-abdominal sepsis, and acute lung injury, and a promising therapeutic target of severe sepsis [12]. Wang et al. found that HMGB1 activated the inflammatory response upon release into the extracellular milieu from necrotic cells and activated macrophages in patients with accompanying organ failure [18]. We examined WBC differential cell counts, including neutrophils; however, we did not find a significant relationship between HMGB-1 levels and neutrophil numbers (data not shown). HMGB-1 may be related to an increase in the recruitment of both neutrophils and monocytes.

It has also been reported that HMGB-1 may mediate various cellular responses, including attachment to Toll-like receptors (TLRs), chemotactic cell movement, and release of proinflammatory cytokines (TNF-α, IL-1β, IL-1α, IL-6, and macrophage inflammatory protein) [12, 18-21]. The presence of an active release mechanism and receptor/signalling system for HMGB1 suggests the physiological significance of this biomolecule.

Our results are in line with a recent report indicating that circulating HMGB-1 concentrations were elevated in both uncomplicated pneumonia and pneumonia with severe sepsis, and much higher in nonsurvivors [22]. These and the present results support the importance of HMGB-1 in the progression of severe lung damage due to viral and bacterial synergic effects and suggest a potential role as a predictor of severe pneumonia.

We followed some patients and examined the time course of their serum levels of HMGB-1. Unfortunately, we did not find significant relationships between HMGB-1 and any clinical findings, including body temperature, heart rate, respiratory rate, WBC count, and CRP level (data not shown). However, we found a decrease in HMGB-1 consistent with improvement of pneumonia.

Although the relationship between serum levels of HMGB-1 and the bacterial and/or viral loads is critical, we could not examine bacterial and viral loads in blood and lung

samples. Our previous study [5] suggested that viral titres were not increased, but bacteria were prolific in lungs of mice with secondary bacterial pneumonia. Therefore, bacterial numbers may be correlated with serum levels of HMGB-1. We will next try to measure the HMGB-1 levels in our mice model [5] using an ELISA system for mice.

In conclusion, we studied immunological mediator molecules, including HMGB-1, IL-6, RANTES, and sICAM-1, and found significant elevations of HMGB-1 and a correlation between WBC count and HMGB-1 in sera from patients with bacterial pneumonia co-infected with influenza virus. HMGB-1 may serve as a useful indicator of severity and a new therapeutic target in patients with severe bacterial pneumonia co-infected with influenza virus.

REFERENCES

1 Mandell LA, Wunderink R, Anzueto A, Bartlett JG, Campbell GD, Dean NC, et al; Infectious Diseases Society of America; American Thoracic Society. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis. 2007; 44:S27-72

2 Woodhead M, Blasi F, Ewig S, Huchon G, Leven M, Ortqvist A et al. Guidelines for the management of adult lower respiratory tract infections. Eur Respir J. 2005; 26:1138-1180

3 Seki M, Hashiguchi K, Kosai K, Higashiyama Y, Yanagihara K , Kurihara S, et al. A patient with fulminant primary influenza pneumonia which developed into secondary bacterial pneumonia. Acta Medica Nagasakiensia, 2006; 51:121-124

4 Seki M, Kosai K, Yanagihara K, Higashiyama Y, Kurihara S, Izumikawa K, et al. Disease severity in patients with simultaneous influenza and bacterial pneumonia. Intern Med. 2007; 46:953-958

5 Seki M, Yanagihara K, Higashiyama Y, Fukuda Y, Kaneko Y, Ohno H, et al. Immunokinetics in severe pneumonia due to influenza virus and bacteria coinfection in mice. Eur Respir J. 2004; 24:143-149

6 Seki M, Higashiyama Y, Tomono K, Yanagihara K, Ohno H, Kaneko Y, et al. Acute infection with influenza virus enhances susceptibility to fatal pneumonia following Streptococcus pneumoniae infection in mice with chronic pulmonary colonization with Pseudomonas aeruginosa. Clin Exp Immunol. 2004; 137:35-40

7 Abramson JS, Giebink G, Mills EL, Quie PG. Polymorphonuclear leukocyte dysfunction during influenza virus infection in chinchillas. J Infect Dis. 1981; 143:836-845

8 Abramson JS, Lyles D, Heller KA, Bass DA. Influenza A virus-induced polymorphonuclear leukocyte dysfunction. Infect Immun. 1982; 37:794-799

9 Parrish W, Ulloa L. High-mobility group box-1 isoforms as potential therapeutic targets in sepsis. Methods Mol Biol 2007; 361:145-162

10 Mantell LL, Parrish W, Ulloa L. Hmgb-1 as a therapeutic target for infectious and inflammatory disorders. Shock. 2006; 25:4-11

11 Yanagihara K, Otsu Y, Ohno H, Higashiyama Y, Miyazaki Y, Hirakata Y, Tomono K, Kadota J, Tashiro T, Kohno S. Clinical characteristics of pneumonia caused by penicillin resistant and sensitive Streptococcus pneumoniae in Japan. Intern Med. 2004; 43:1029-1033

12 Yamada S, Maruyama I. HMGB1, a novel inflammatory cytokine. Clin Chim Acta. 2006; 375:36-42

13 Yamada S, Inoue K, Yakabe K, Imaizumi H, Maruyama I. High mobility group protein 1 (HMGB1) quantified by ELISA with a monoclonal antibody that does not cross-react with HMGB2. Clin Chem. 2003; 49:1535-1537

14 Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE, Fanning TG. Initial genetic characterization of the 1918 "Spanish" influenza virus. Science. 1997; 275:1793-1796

15 Reid AH, Fanning TG, Hultin JV, Taubenberger JK. Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. Proc Natl Acad Sci U S A. 1999; 96:1651-1656

16 Chan MC, Cheung CY, Chui WH, Tsao SW, Nicholls JM, Chan YO, Chan RW, Long HT, Poon LL, Guan Y, Peiris JS. Proinflammatory cytokine responses induced by

influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir Res. 2005; 6:135-148

17 McCullers JA Effect of antiviral treatment on the outcome of secondary bacterial pneumonia after influenza. J Infect Dis. 2004; 190:519-526

18 Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. HMG-1 as a late mediator of endotoxin lethality in mice. Science. 1999; 9:248-251

19 Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem. 2004; 279:7370-7377

20 Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol. 2006; 290:C917-924

21 Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, et al. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med. 2000; 192:565-570

22 Angus DC, Yang L, Kong L, Kellum JA, Delude RL, Tracey KJ, et al. Circulating high-mobility group box 1 (HMGB1) concentrations are elevated in both uncomplicated pneumonia and pneumonia with severe sepsis. Crit Care Med. 2007; 35:1061-1067

FIGURE LEGENDS

Figure 1

Concentration of HMGB-1 in sera from patients with bacterial pneumonia co-infected with influenza virus and patients with bacterial pneumonia alone. (A): The concentrations of HMGB-1 in sera from co-infected patients [Flu (+)] and from patients with bacteria infection alone [Flu (-)]. Whisker box plots represent the 25^{th} to 75^{th} percentiles of results inside the box; the median is indicated by a horizontal bar across the box; whiskers in each box represent 10^{th} to 90^{th} percentiles. Differences remained significant after Bonferroni's correction. $p<0.01$ (Mann-Whitney U test). (B): Correlation between the white blood cell count and the concentration of HMGB-1 in the sera from patients with bacterial pneumonia co-infected with influenza virus.

Table 1. Clinical characteristics and levels of cytokines in sera from 12 patients with bacterial pneumonia co-infected with influenza virus.

Pt. #	Age	Gender	Complications	Bacteria	Temp (℃)	HR (min)	RR (min)	WBC (mm^3)	CRP (mg/L)	IL-6 (pg/mL)	RANTES (ng/mL)	sICAM-1 (pg/dL)	HMGB-1 (ng/mL)
1	36	M	none	*H. influenzae*	37.5	58	20	3330	1.7	25.0	61.3	125.0	38.0
2	60	M	COPD, old Tbc	*P. aeruginosa*	36.9	61	20	10270	12.7	53.7	67.8	115.4	54.1
3	44	M	Kidney stone	*S. pneumoniae*	37.7	60	18	6600	0.4	31.4	60.6	99.0	34.5
4	32	F	none	*H. influenzae*	36.4	60	18	6260	1.4	44.2	49.5	74.7	28.4
5	28	M	none	*H. influenzae*	36.9	88	18	3620	1.7	45.6	73.9	107.0	20.2
6	26	F	none	*H. influenzae*	38.0	72	20	8700	1.1	33.9	57.4	117.5	42.5
7	80	M	old Tbc, HT	*S. aureus*	36.8	88	28	8700	13.8	186.8	53.7	91.7	65.6
8	90	F	COPD	*S. aureus*	38.1	90	24	6510	10.3	98.3	46.8	130.2	55.1
9	30	F	none	*S. pneumoniae*	39.0	60	18	6600	1.7	24.4	57.1	133.8	50.2
10	71	F	Heart	*H. influenzae*	37.1	86	22	10400	1.6	38.3	58.4	110.0	80.5
11	65	F	old Tbc	*H. influenzae*	38.5	88	20	9300	1.3	41.4	68.9	111.9	30.0
12	50	F	none	*S. pneumoniae*	38.0	66	20	6740	7.5	89.1	58.6	83.2	49.3

H. influenzae, *Haemophilus influenzae;* COPD, chronic obstructive lung disease*;* Tbc, tuberculosis; *P. aeruginosa*, Pseudomonas aeruginosa; tumor, malignant neoplasm; *S. pneumoniae*, *Streptococcus pneumoniae*; *S. aureus*, *Staphylococcus aureus*;, Xp, Chest X-ray; HR, heart rate; RR, respiratory rate; WBC, white blood cells count; CRP, C-reactive protein; IL-6, interleukin-6; RANTES, regulated on activation, normal T-cell expressed and secreted; sICAM-1, soluble intercellular adhesion molecule-1; HMGB-1, high mobility group box chromosomal protein-1

Table 2. Levels of immuno-modulators in sera from patients with bacterial pneumonia co-infected with influenza virus [Flu (+)] and patients with bacterial pneumonia alone [Flu (-)].

	Flu (-) (n=18)	Flu (+) (n=12)	p Value
IL-6 (pg/ml)	30.9 ± 11.3	59.4±46.4	p<0.05
RANTES (ng/ml)	56.6 ±12.1	59.5 ±7.8	NS
sICAM-1 (pg/dl)	113.4 ±19.2	108.3 ±18.3	NS

Data are expressed as the mean ± SD
p<0.05 between groups, Mann-Whitney U rank test.
NS= not significant, Flu (+): Patients with bacteria and influenza virus co-infected, Flu (-): Patients with bacteria infection alone.

Fig. 1. Concentration of HMGB-1 in sera from patients with bacterial pneumonia co-infected with influenza virus [Flu (+)]and patients with bacterial pneumonia alone [Flu (-)].