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Original Article

Expression and DNA Microarray Analysis of a Platelet Activating Factor-Related Molecule in Severe Pneumonia in Mice Due to Influenza Virus and Bacterial Co-Infection

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SUMMARY: Platelet-activating factor (PAF) is a critical mediator of severe inflammatory diseases such as pneumonia, and the PAF-receptor (PAFR) is known to be an anchor for *Streptococcus pneumoniae* attachment to lung epithelial cells. We conducted a DNA microarray analysis to detect critical factors that mediate fulminant pneumonia due to influenza virus and *S. pneumoniae* co-infection in mice. Among the factors detected, levels of PAF-acetyl hydrolase (PAF-AH), which functions as inactivated PAF, were significantly increased, and PAFR was expressed in co-infected mouse lungs, as compared to the respective levels in mice infected with either *S. pneumoniae* or virus alone. Significantly elevated PAF-AH enzymatic activity was observed in the co-infected mouse lung, suggesting that co-infection activated PAF-related factors. These findings suggest that PAF and related molecules play important roles in fulminant pneumonia due to influenza virus infection, especially when severe bacterial pneumonia is complicated by co-infection with influenza virus.

INTRODUCTION

Influenza virus infection is a major respiratory disease worldwide, and pneumonia is one of the most important complications associated with influenza (1-5). During influenza season, excessive mortality in the elderly is commonly due to such complications.

Influenza infection-associated pneumonia is classified as either primary viral or as secondary/mixed bacterial pneumonia (3,5-7). The latter type is more frequently seen than primary viral pneumonia and usually develops late in the course of the disease (5-7). The Gram-positive bacterium *Streptococcus pneumoniae* is among the major causes of severe cases of influenza-related pneumonia, although about 40% of individuals asymptomatically harbor pneumococcus in the nasopharynx (7,8).

Our previous study of mice showed fulminant pathological changes in the lungs of mice inoculated with influenza virus followed by *S. pneumoniae* infection 2 days later. Certain critical immune responses (e.g., cytokine and chemokine release and Toll-like receptor [TLR] expression) were found to be increased in these mice, although only moderate pneumonia/bronchitis was observed in those animals infected with either influenza virus or *S. pneumoniae* alone (9). In addition, chronic *Pseudomonas aeruginosa* infection in mice is known to be exacerbated by the influenza virus, whereas decreased neutrophil function due to viral infection possibly induces the lethal pneumococcal pneumonia observed in such mice (10). These results are suggestive of synergistic effects of co-infection with influenza virus and bacteria. Moreover, specific immunological reactions appear to mediate such severe pneumonia.

Among the immunological molecules studied in the context of severe pneumonia, platelet-activating factor (PAF) and related molecules are involved in bacterial pneumonia during post-influenza pneumonia. McCullers and Rehg investigated the potential role of the PAF receptor (PAFR) in pneumococcal pneumonia following influenza A infection (11), and van der Sluijs et al. reported that the PAFR is involved in the host defense against *S. pneumoniae* during post-influenza pneumonia in PAFR-knockout mice (12).

Here, we conducted a DNA microarray analysis in order to detect those immunological molecules specifically related to the development of fulminant pneumonia caused by influenza virus and *S. pneumoniae* co-infection in mouse models, and we compared our findings with those obtained with mice infected with either *S. pneumoniae* or influenza virus alone. We also identified and further analyzed the roles of PAF-related compounds among 15 that were specifically induced in co-infected mice.

MATERIALS AND METHODS

Virus, bacteria, mouse infection, and sampling: A mouse-adapted A-strain influenza virus (strain A/PR8/34: H1N1 type) and/or *S. pneumoniae* (penicillin-resistant *S. pneumoniae*-187 serotype 19 isolated at our university) was inoculated into 6-week-old male CBA/J mice (specific pathogen-free) as previsouly described (9). Briefly, each mouse was inoculated intranasally with 50 μL of influenza virus (1×10^4 plaque-forming units/mL), followed by intranasal inoculation with 50 μL of *S. pneumoniae* (1×10^8 CFU/mL) 2 days later. Saline was used for intranasal inoculation of mock-infected mice. The lungs were harvested as biological samples at 48 h

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after *S. pneumoniae* infection. Comparisons of microarray patterns of the lung tissue harvested from co-infected mice and those from the lungs of mice infected with *S. pneumoniae* alone were conducted. In addition, comparisons of microarray patterns were also performed to identify differences between mice with the co-infection and those infected with influenza virus alone.

RNA isolation: Total RNA was extracted from lung homogenates using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Lungs were disrupted in 1 mL of serine using a glass tissue homogenizer (Takashima, Tokyo, Japan) for 60 s in an ice-cold water bath, followed by phenol-chloroform extraction and isopropyl alcohol precipitation. We isolated mRNA from the total RNA using a MicroPoly(A) Pure kit (Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's instructions.

Microarray analysis: To clarify the gene expression profiles in the context of severe pneumonia, we performed a DNA microarray analysis using IntelliGene™ Mouse CHIP Set I (Takara Shuzo, Kyoto, Japan), in which about 600 mouse-related genes, about 300 expressed sequence tags, and 10 control housekeeping genes (see http://www.takara.co.jp/bio/goods/new/new6/new6-7.htm) are spotted on 4 glass plates. The isolated mRNA was evaluated by electrophoresis in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif., USA) and was further purified, and then the concentration was measured. A fluorescent probe was synthesized by the incorporation of Cy3- or Cy5-dUTP using 6.6 μg of the above mRNA as a template and 50U AMV reverse transcriptase (Takara Shuzo). We added 50 pg of lambda A into the reaction mixture as an internal control. Cy3- and Cy5-labeled probes prepared using mRNA isolated from co-infected or singly-infected mice, respectively, were added to the reaction buffer (6 × SSC/0.2% SDS and 5 × Denhardt's solution, containing carrier DNA). The mixture was hybridized to the cDNA CHIP at 65°C for 14 h. The CHIP was washed once with 2 × SSC/0.2% SDS at 55°C for 5 min, once at 65°C for 5 min, and once with 0.05 × SSC at room temperature. The hybridized CHIP was visualized and quantified using an Affimetrix (Woburn, Mass., USA) 428 Array Scanner and ImaGene software Ver.4.2 (Bio-Discovery, Los Angeles, Calif., USA). The TIFF files created by the Affimetrix 418 Array Scanner were colored using Adobe (Mountain View, Calif., USA) Photoshop software. These microarray analyses were performed twice.

Measurement of PAF-acetyl hydrolase (PAF-AH) activity: PAF-AH activity in aqueous lung extracts was measured using a PAF Acetylhydrolase Assay Kit (Cayman Chemical, Ann Arbor, Mich., USA). Lung homogenates were diluted 10-fold with 0.1 M Tris-HCl (pH 7.2) containing heparin and separated by centrifugation for 15 min at 10,000 × g at 4°C. Supernatants were collected and assayed according to the attached protocol (13,14). The concentrations were multiplied by the dilution factor.

RT-PCR analysis of PAFR expression: We performed reverse transcription (RT)-polymerase chain reaction (PCR) using the First Strand & Detection Kit (Life Technology, Gaitherberg, Md., USA). Mouse PAFR and hypoxanthine phosphoribosyltransferase (HPRT) served as the internal control, and cDNAs were respectively amplified using the following specific primers: mouse PAFR, forward 5′-CCT GAT CAC CCT CCT ACT GT-3′ and reverse 5′-GAT GAT GAA TAC CGC CAA GA-3′ (530 bp); and mouse HPRT, forward 5′-AAG CAG TAC AGC CCC AAA AT-3′ and reverse 5′-CAT AGT GCA AAT CAA AAG TC-3′ (392 bp). Each factor was amplified by 25 PCR cycles of 1 min each at 94°C, 55°C, and 72°C. The signal intensity of each band was semi-quantified using an Alpha Imager (Alpha-Innotech Co., San Leandro, Calif., USA).

Western blots to detect PAFR expression: We detected PAFR in the lung homogenates by Western blot analysis using goat anti-PAFR antibody (1:500; Santa Cruz Technology, Santa Cruz, Calif., USA) and horseradish peroxidase-conjugated anti-goat IgG (1:50,000, Santa Cruz Technology) as the secondary antibody.

In brief, proteins in the lung homogenates were separated by SDS gel electrophoresis and were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass., USA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline (pH 7.2 - 7.4) containing 0.05% Tween-20 (TBS-T), incubated with first antibody overnight at room temperature, washed with TBS-T, and incubated with second antibody for 1 h at room temperature. Positive signals were visualized using ECL (GE Healthcare Bioscience, Tokyo, Japan).

Statistical analysis: All data are expressed as means ± SD and were analyzed using Stat View software (Abacus Concepts, Cary, N.C., USA). The significance of differences among groups was examined using ANOVA followed by Tukey's or Dunnett's tests. A *P*-value of below 0.05 was considered to indicate a statistically significant difference.

RESULTS

Microarray analysis: Scatterplots of Cy3/Cy5 of all genes on the arrays were evaluated to determine procedural accuracy. The corresponding images showing expressed genes from either co-infected or singly-infected lungs were superimposed onto each other. Green spots indicate downregulated genes under conditions of co-infection, red indicates upregulation, and yellow (i.e., mixture of red and green) indicates genes that were equally expressed in cases of co-infection, as compared to levels observed in singly-infected lungs.

The Cy3/Cy5 ratio was adjusted/normalized, and it was found the detected genes, especially all housekeeping genes, were within twofold (data not shown). The high correlation and quality of the results indicated that the DNA microarray analysis was very reliable. A more than twofold difference was considered significant.

Table 1 lists those genes that were specifically induced in co-infected lungs, and compares the results with those obtained from *S. pneumoniae*-only infected lungs (fold differences at left). Among the 15 genes detected, we focused on PAF-AH because PAF and the related PAFR are considered critical factors in post-influenza pneumonia (11,12). Significant levels of PAF-AH were also expressed in co-infected lungs, as compared to those in influenza virus-only infected lungs (Table 1, fold differences at right).

Only three genes (casein kinase-1, cyclin D1, adenylosuccinate lyase) were downregulated in co-infected lungs, as compared with results obtained from lungs infected with *S. pneumoniae* alone.

PAF-AH activity: The PAF-AH enzyme converts PAF to biologically inactive lyso-PAF (14). We therefore analyzed PAF-AH activity in mouse lung homogenates (Figure 1). PAF-AH activity was significantly higher in lungs co-infected with *S. pneumoniae* alone. In addition, greater PAF-AH was

Table 1. Induced genes in co-infected lungs analyzed by DNA microarray

Gene name	GenBank accession no.	Fold differences*
TATA-box binding protein	U63933	6.1 (2.3)
G protein coupled receptor	NM 010336	5.8 (3.3)
Platelet-activating factor acetyl hydrolase (PAF-AH)	U577747	5.3 (4.3)
Conserved helix-loop-helix ubiquitous kinase	U12473	5.0 (2.0)
Cyclin-dependent kinase 2 (CDK2)	U63337	3.5 (2.5)
CD19 antigen	NM 009844	3.4 (4.2)
Transforming growth factor beta (TGF-b) induced transcriptase	NM 009365	3.3 (1.1)
Ubiquitin activating enzyme (E1C)	AF077330	3.2 (1.2)
UDP-glucuronosyltransferase 1 family member 1	U16816	3.2 (none)
Angiotesinogen	AF045887	2.8 (none)
Proline-serine-threonine phosphatase interacting protein	U87814	2.8 (none)
E2F binding protein 1	AF015948	2.5 (1.2)
Nucleotide binding protein 1	NM 011955	2.4 (none)
Coagulation factor VII	U44795	2.1 (1.1)

* Fold differences indicated ratio of the genes expression in co-infected lungs, compared with those in single (bacteria or virus only) infected lungs. Left, bacteria only, and right ( ), virus only infection.

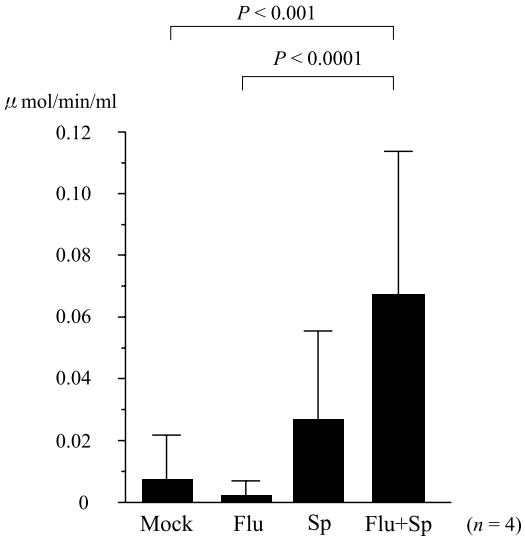

Fig. 1. Activity of platelet-activating factor acetyl hydrolase (PAF-AH) in lungs of mice infected with or without influenza virus and/or *S. pneumoniae*. Enzyme activities of PAF-AH were increased in co-infected lungs. Mock, mock-infected lungs; Flu, influenza virus singly infected lungs; Sp, bacteria singly infected lung; Flu + Sp, influenza virus and bacteria co-infected lungs. Results are representative of four repeated experiments. Values of $P < 0.05$ are considered to indicate a statistically significant difference ($n = 4$).

observed in co-infected lungs than in either influenza virus-infected only or mock-infected lungs.

Expression and detection of PAFR in co-infected lungs: We further analyzed PAFR expression in the mouse lung. Expression levels of PAFR mRNA were very low and difficult to determine in the lungs of infected with bacteria alone. However, PAFR expression increased significantly in co-infected lung homogenates (Figure 2A, B). In addition, higher levels of PAFR expression were also observed in these samples than in either influenza virus-infected alone or mock-infected samples.

We then analyzed PAFR secretion by Western blotting. Negligible PAFR was detected in mock-infected mouse lungs. PAFR secretion levels were also very low in the lungs with influenza virus and in those infected with only *S. pneumoniae*. However, markedly high levels of PAFR expression were observed in co-infected lungs (Figure 3).

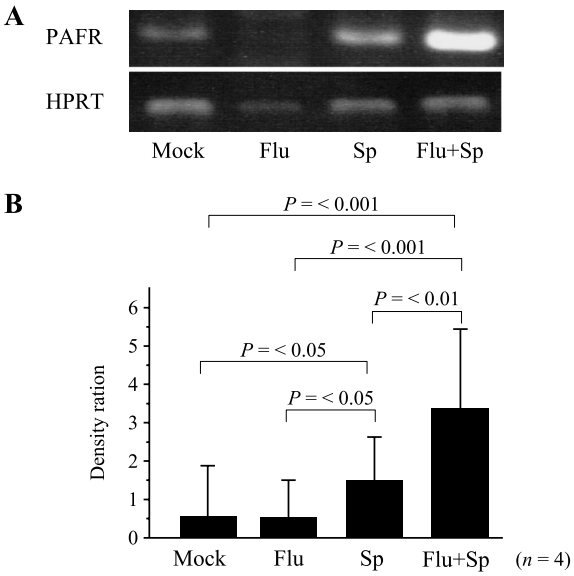

Fig. 2. (A) Expression levels of platelet-activating factor receptor (PAFR) in mouse lungs infected with or without influenza virus and/or bacteria. Samples were analyzed by RT-PCR with hypoxanthine phosphoribosyltransferase (HPRT) as internal control. Mock, mock-infected lungs; Flu, influenza virus singly infected lungs; Sp, bacteria singly infected lung; Flu + Sp, influenza virus and bacteria co-infected lungs. Results are representative of four repeated experiments. (B) The relative densities. Values of $P < 0.05$ are considered to indicate a statistically significant difference ($n = 4$).

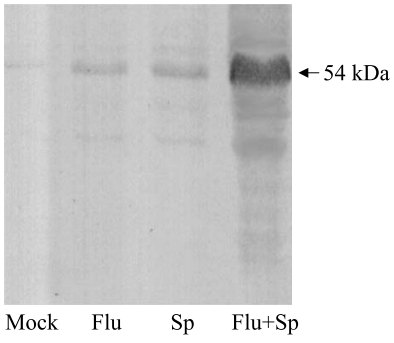

Fig. 3. Western blotting to analyze PAFR secretion. Mock, mock-infected lungs; Flu, influenza virus singly infected lungs; Sp, bacteria singly infected lung; Flu + Sp, influenza virus and bacteria co-infected lungs. Results are representative of six repeated experiments.

DISCUSSION

Influenza pneumonia following a bacterial infection is frequently observed and poses a severe clinical complication during influenza season. Secondary/mixed bacterial pneumonia with influenza virus infection ofen progresses in terms of severity with lung hemorrhage, and resembles acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) status. Specific immunological reactions and synergic interaction between influenza virus and bacteria have been suggested in the literature (9,10,15,16).

Here, we used a DNA microarray analysis to examine the molecules that mediate the type of fulminant bacterial pneumonia that arises due to co-infection with influenza virus in mice. In our experimental mice, co-infected mice showed shorter survival times due to the development of more severe pneumonia, as compared to mice infected with either influenza or *S. pneumoniae* only. In addition, bacterial numbers were relatively high in the lungs of co-infected mice, as compared to those in the lungs infected with *S. pneumoniae* only, despite our previously reported findings of similar viral titers in the lungs of both the co-infected and only influenza-infected groups (9).

The expression of hundreds of genes can be simultaneously analyzed using DNA microarrays, which facilitates the differential expression monitoring of a large number of activated or suppressed genes under various biological conditions. DNA microarrays are thus useful tools for screening, and the information accumulated by the use of this technology stands to be helpful for elucidating the regulatory networks that participate in severe pneumonia (17,18).

Here, we compared gene expression profiles in co-infected lungs and in those infected with bacteria alone, and we detected PAF-related molecules such as PAF-AH. Fifteen genes, including transcriptional factors and receptors, were specifically induced in the co-infected lungs, yet here we focused solely on PAF-AH.

After stimulation, various cells synthesize the biologically active phospholipid PAF and induce inflammation mediated via inflammatory cells such as macrophages and neutrophils. PAF is known to be produced in cases of severe pneumonia due to *S. pneumoniae* infection (8). Additionally, the PAFR serves as an anchor for *S. pneumoniae*, thus facilitating-infection; moreover, the attachment of bacterial phosphorylcholine to PAFR enhances adhesion, which is associated with invasion of endothelial, epithelial, and PAFR-transfected cells (19). In post-influenza pneumonia induced by *S. pneumoniae*, PAF involvement has been confirmed using PAFR-knockout mice and a PAFR antagonist (11,12).

Unfortunately, we did not perform a DNA microarray analysis using non-infected (mock/control) lungs, but rather performed the present analysis using influenza virus only infected mice. In this study, we found significant upregulation of PAF and related molecules. In addition, we measured PAF-AH secretion/activity in the lungs of either virus-infected alone or mock-infected mice, but only very low levels of secretion and activity of PAF-AH were observed, in spite of the statistically significant increase in PAF-AH activity observed in co-infected lungs. These findings suggest that the present DNA microarray analysis was useful and reliable. Furthermore, the present results highlight the importance of PAF and related factors in severe, influenza-related bacterial pneumonia.

The present results confirmed the significant expression and secretion of PAFR and PAF-AH in co-infected mouse lungs, and levels of the latter were also found to be elevated in this group. PAF converted by PAF-AH is degraded and biologically inactive; however, PAF-AH activity has been reported to be elevated in the bronchoalveolar lavage fluid from patients with ARDS (20). The presence of PAF-AH mRNA in alveolar macrophages suggests active synthesis in the lungs of patients with ARDS. Changes in levels of PAF activity in the lungs of ARDS patients might regulate the inflammation caused by PAF and related oxidized phospholipids generated during the inflammatory response.

The roles of PAF and PAF-AH have remained controversial, but migration failure was prevented and bacterial clearance was found to be more efficient in PAFR-deficient mice and in mice pretreated with a PAFR antagonist and subjected to lethal sepsis and neutrophil depletion (21). Systemic inflammation (low serum cytokine levels) is reduced, plasma nitrate levels are lower, and survival rates are higher in these animal models. Mice deficient in PAFR are resistant to pneumococcal infection, and the rate of pneumolysin expression, an important virulence factor of *S. pneumoniae*, is accelerated during PAF-induced acute injury in the mouse lung (22,23). Thus, a blockade of the PAFR might prevent the onset of severe sepsis and pneumonia.

However, in post-influenza pneumonia models, McCullers and Rehg found that mice infected with influenza virus and then treated with a PAFR antagonist displayed enhanced outgrowth of pneumococci, even though van der Sluijs et al. observed the development of less severe pneumonia using a similar protocol and PAFR-knockout mice (11,12). The discrepancy between results was accounted for as reflective of differences in *S. pneumoniae* strains. We also performed PAF-blocking experiments using a PAF-antagonist in influenza-infected and *S. pneumoniae* co-infected mice, and we found a slight increase in the survival times of these mice treated by a PAF-antagonist; however, statistically significant longer survival was not observed (data not shown). Further investigation into the roles of PAF/PAF-AH in post-influenza pneumonia will still be necessary.

In conclusion, we performed a microarray analysis to detect key molecules related to the development of severe pneumonia caused by co-infection with influenza virus and bacteria, and we focused in particular on PAF-AH. Expression levels of the PAFR and PAF-AH activity were both elevated in co-infected mouse lungs, suggesting that PAF-related molecules are indeed involved in the pathogenesis of such severe pneumonia. Although the functions and roles of each PAF-related molecule remain unclear, it is possible that the PAFR is associated with bacterial adherence and with several mechanisms of action that influence pneumonia severity.

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