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Research Paper

C-Reactive Protein Mediating Immunopathological Lesions: A Potential Treatment Option for Severe Influenza A Diseases

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ABSTRACT

Severe influenza diseases with high mortality have been frequently reported, especially in those patients infected with avian influenza A (H5N1, H7N9 or H10N8) or during a pandemic. Respiratory distress, which is attributed to alveolar damage associated with immunopathological lesions, is the most common cause of death. There is a wealth of information on pathogenesis or treatment options. In this study, we showed that high levels of C-reactive protein (CRP) were induced and correlated with complement activation in patients infected with severe influenza A (H5N1, H7N9 or H10N8), and higher levels were induced in fatal patients than in survivors. CRP treatment enhanced the phagocytosis of monocytes THP-1 to H5N1 virus as well as the expression of proinflammatory cytokines or apoptosis-associated genes in THP-1 cells or pneumocytes A-549 respectively. CRP may link to proinflammatory mediators contributing to activation of complement and boosting inflammatory response in severe influenza infections. Compound 1,6-bis(phosphocholine)-hexane improved the severity and mortality of mice infected with lethal influenza virus significantly. These observations showed that CRP is involved in deterioration of severe influenza diseases, and indicated a substantial candidate molecule for immunotherapy of severe influenza diseases.

1. Introduction

Influenza comprises a substantial portion of morbidity and mortality caused by acute respiratory infections, which are the fourth leading cause of death and the second highest cause of years of life lost (Organization, 2014; Radin et al., 2012; Nair et al., 2011). Severe influenza diseases with high mortality have been frequently reported, especially in those patients infected with avian influenza (AI) A (H5N1, H5N6, H7N9 or H10N8) viruses or during a pandemic (Organization, 2015; Ortiz et al., 2013; Chen et al., 2014). Severe AI or pandemic patients present with rapidly progressive pneumonia, leading to acute respiratory distress syndrome or the complication of multiple organ failure (Gao et al., 2013a; Pan et al., 2016; Chen et al., 2014; Investigators et al., 2009). Respiratory distress is the most common cause of death in patients infected by these viruses (Liem et al., 2009; Gao et al., 2013a). Autopsy or postmortem biopsy studies showed that extensive diffuse alveolar damage is the most consistent finding, and the virus predominantly infected lung parenchyma in H5N1, H5N6, and H7N9 patients or fatal cases infected by influenza pandemic viruses (Guo et al., 2014; Gao et al., 2016; Shieh et al., 2010).

In terms of therapy, other than lung protective ventilation for support for oxygenation, antiviral treatment is an important component to manage those patients infected with these viruses (Gao et al., 2013a; Yu et al., 2008; Zhang et al., 2009). However, influenza viruses mutate readily, and produce drug-resistant strains or unmanageable stains using vaccine (Govorkova et al., 2013; Hu et al., 2013; Shore et al., 2013). In addition, in case of lethal influenza infection, antiviral treatment is needed early to block virus replication and prevent triggering dysregulation of immune response, thereby abrogating the immunopathology (Wong et al., 2011). Once triggered, the immune-mediated tissue damage possibly presents limited sensitivity to antiviral agents (Yen et al., 2005; Govorkova et al., 2009).

C-reactive protein (CRP), a pentraxin, is a marker of inflammation that has been extensively used in clinical practice. CRP is involved in the innate immune response by attaching to microorganisms and damaged cellular components via phosphocholine. This leads to complement activation and phagocytosis. Excessive host immune response including complement activation has been shown to play a critical role on pathogenesis of severe influenza infections (US, 2008; Gao et al., 2013b). Activation of the complement system has been implicated

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in the development of acute lung diseases induced by highly pathogenic influenza viruses (Garcia et al., 2013; Sun et al., 2013, 2015). Recent studies have brought up the idea of CRP to be not only a systemic marker of inflammation but also a mediator in inflammation faci (Thiele et al., 2015), and indicated that high levels of CRP is a high risk factor of fatality in H7N9 patients (Cheng et al., 2015; Wu et al., 2016). However, its exact physiological roles remain largely unknown, especially in severe influenza diseases. In this study, we characterized the role of CRP on pathogenesis of severe infections by influenza A (H5N1, H7N9 or H10N8) viruses, and indicated a candidate molecule for immunotherapy of severe influenza infection.

2. Materials and Methods

2.1. Influenza Patients and the Assessment of Patient Sera

Archived serum samples were obtained from local Centers for Diseases Control and Prevention (CDC) participating in the National Influenza Surveillance Network. H5N1, H7N9, H10N8, or H1N1 pdm09 infection was confirmed by reverse transcription polymerase chain reaction (RT-PCR) or viral isolation according Chinese influenza surveillance guideline. No samples were obtained from patients with presumptive (clinical diagnosis). Control samples were set from 10 normal donors from poultry workers. The sera CH50 levels were detected by MicroVue CH40 Eq EIA kit (Quidel, USA) per the kit's protocol. The sera CRP and C3 levels were tested by Bejing CIC clinical laboratory using turbidimetric inhibition immuno assay.

2.2. Viral Titration

The influenza viruses used in this study were titrated by a TCID50 (50% tissue culture infectious dose) in MDCK cells. Briefly, 100 μL/well of MDCK cells (3×10^4 cells/mL) were seeded one day before infection in 96-well microtiter plates. Serial semi-logarithmic dilutions of each virus or samples were made with Dulbecco modified Eagle medium containing 1% bovine serum albumin and 2 μg/mL TPCK-treated trypsin from 10^{-2} to 10^{-7}. Dilutions of each virus or sample were added to MDCK cells (4 wells for each dilution, 100 μL/well). The cells were incubated for 48 h at 37 °C. The contents of each well were tested for hemagglutination by incubating 50 μL of the tissue culture supernatant with 0.5% turkey erythrocytes. The TCID50 was calculated according to the Reed and Muench method. For mouse lung tissue processing, in brief, whole lung tissues from each mouse were homogenized in 2 mL of phosphate buffered saline (PBS). The supernatant was sampled after centrifugation at 3000 rpm for 15 min at 4 °C.

2.3. Viral Infection In Vitro

The infection was performed by seeding the paired A-549 and THP-1 cell at a 1:1 cell ratio (100,000 cells each) onto a Transwell insert co-culture system (SI Appendix Fig. S4). A-549 were seeded onto the lower chamber of a 12-well plate and THP-1 onto Millicell hanging cell culture inserts (0.4 μm pore size; Costar, USA) in 0.5 mL of MEM (supplemented with 1% BSA and 1% antibiotic-antimycotic solution) with or without CRP (20 mg/L, BiosPacific, USA) at 37 °C. Another group was set as that CRP receptor blockers (100-fold diluted monoclonal antibodies against FcyR-I and FcyR-II; R&D, USA) were added into CRP-treated cells. The A-549 cells were infected with 0.1 multiplicity of infection (MOI) of A/Guangxi/1/2010(H5N1) virus. Four duplicates were set for each infection or MOCK group. The A-54ss9 or THP-1 cells were harvested for quantitative RT-PCR or immunochemistry detections at 6 h or 24 h post of infection, respectively. All steps were performed in biosafety level - 3 containment laboratory.

2.4. Mouse Infection

All animal studies were performed according to guidelines approved by the Investigational Animal Care and Use Committee of the National Institute for Viral Diseases Control and Prevention of the China CDC. We performed viral challenge by i.n. inoculation of 10^5 TCID50 of A/PR/8/34 (10-fold 50% lethal dose) to anesthetized 6- to 8-week adult female C57 BL/6 mice in 50 μL PBS. Intraperitoneal administration of 1,6-bis PC or/and peramivir (Abmole Bioscience, USA) was started 48 h after virus infection. A total of 4 types of treatment regimens (0.1 mM 1,6-bis PC, 0.02 mM 1,6-bis PC, 3 mg peramivir, and 3 mg peramivir plus 0.1 mM 1,6-bis PC per kg each day) were administered until day 7 post-infection. Compound 1,6-bis PC was synthesized in SKS Chem according to a previous report (Pepys et al., 2006). All compounds were diluted in TC (0.01 M Tris plus 0.14 M NaCl and 0.002 M $CaCl_2$, pH 8.0) buffer. If the mice lost over 25% of their initial body weight, they were humanely euthanized and necropsied.

2.5. Immunochemistry Assay

An immunochemistry test for detection of influenza A antigen was performed on the slides with a THP-1 smear by using a polymer-based colorimetric indirect peroxidase method (ZSBio, China). The THP-1 cells were fixed using 80% acetone. A mouse monoclonal antibody against the nucleoprotein of influenza A was used (Serotec, UK). For controls, we used an antibody against nucleoprotein of influenza B virus in place of the primary antibody.

2.6. RNA Extraction and Quantitative RT-PCR

RNA was extracted from THP-1 or A549 cells using automated RNA extraction system QIAsymphony RGQ (Qiagen, Germany) with QIAsymphony RNA extraction kits as per the kit's protocol. To quantify the influenza viral load, proinflammation cytokine (IL-6 and IP-10), apoptosis-associated (TRAIL and FAS) gene, C5a receptor (C5aR1 and C5aR2) and CRP receptor (FCGR2 and FCGR3) mRNA levels, a quantitative real-time RT-PCR was performed on a real-time PCR detection system (Agilent Technologies Inc., Santa Clara, CA). The primer and probe sets were described in our previous report (Gao et al., 2013b) or from commercial products (Hs02375669, Hs01906226, Hs04206243, Hs04211858; Life Science Technologies, USA).

2.7. Histopathology and Immunohistochemistry

Routine hematoxylin and eosin staining was used for histopathology evaluation. For immunohistochemistry, 4 μm deparaffinized formalin fixed paraffin embedded sections were stained with monoclonal antibody against CRP (Abcam, USA), CD20cy (Dako Denmark), CD8 (Dako Denmark) and complement C5a (Abcam, USA) by using a polymer-based colorimetric indirect peroxidase method (ZSBio, China).

2.8. Mouse CRP, Lactate Dehydrogenase B, and Creatine Kinase-MB Assay

Mouse CRP, lactate dehydrogenase B (LDHB) and creatine kinase-MB (CK-MB) levels in mice sera were determined using enzyme-linked immunosorbent assay according to the manufacturer's instructions (CRP detection kit from R&D system, USA; LDHB and CK-MB detection kits from Dldevelop, China).

2.9. Statistical Analysis

Statistical analysis was performed using Instat software (Version 5.0, GraphPad Prism). Statistical significance was determined by Mann-Whitney *U* test or Kruskal-Wallis, Pearson's correlation or nonparametric *t*-test. Differences were considered significant at $p < 0.05$ with two-tailed testing.

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