Association of Tumor Necrosis Factor-α with Fever and Pulmonary Lesion Score in Pigs Experimentally Infected with Swine Influenza Virus Subtype H1N2

Bongtae KIM1), Kyoung Kyu AHN1), Yooncheol HA1), Yong Hoon LEE1), Duyeol KIM1), Jeong Han LIM2), Sung-Hoon KIM2), Mi-Young KIM3), Kyung-Dong CHO3), Bog-Hieu LEE3) and Chanhee CHAE1)*

1)Department of Veterinary Pathology, College of Veterinary Medicine, Seoul National University, San 56–1, Shillim-Dong, Gwanak-Gu, 151–742 Seoul, 2)College of Oriental Medicine, Kyunghee University, 1 Hoegi-dong, Dongdaemun-ku, 130–701 Seoul and 3)Department of Food and Nutrition, Chung-Ang University, Nae-Ri 72–1, Dae-deok-Myeon, 456–756, Gyeonggi-Do, Republic of Korea

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ABSTRACT. The relationship between tumor necrosis factor (TNF)-α level, body temperature, and pulmonary lesion score was determined in 3-week-old pigs inoculated intranasally with swine influenza virus (SIV) subtype H1N2. The expression of TNF-α was measured in bronchoalveolar lavage (BAL) fluids by enzyme-linked immunosorbent assay and lung tissues by immunohistochemistry. In BAL fluid, TNF-α concentration was maximal at 1 days post-inoculation (dpi), declined markedly by 3 dpi (P<0.05) and steady thereafter. Mean rectal temperatures were above 40°C for the infected groups at 1 dpi and declined markedly by 3 dpi. The body temperatures were correlated with the levels of TNF-α in BAL fluids from pigs experimentally infected with SIV (r²=0.9754, P<0.05). The pulmonary lesion scores were correlated with the means of positive cells by immunohistochemistry for TNF-α (r²=0.9949, P=0.001). The results suggest that the expression of TNF-α protein plays an important role in induction of pulmonary lesion and clinical sign such as fever in SIV infection.

KEY WORDS: fever, H1N2 subtype, swine influenza virus, tumor necrosis factor-α.

Influenza viruses are negative single-stranded RNA viruses that belong to the family Orthomyxoviridae [13]. Influenza viruses are divided into 3 types (A, B, and C) based on antigenicity of the nucleocapsid protein and are subtyped based on the antigenicity of the HA and NA proteins [19]. Recently, the H1 and H3 subtypes, in combination with N1 or N2 subtypes, caused widespread epidemic and endemic among swine population [18]. The causative viruses are type A influenza virus of H1N1, H1N2 and H3N2 subtypes in swine [18].

Swine influenza virus (SIV) is a highly important respiratory disease. Typically, swine influenza is recognized as an acute febrile respiratory disease with high morbidity and low mortality [8]. Nasal and eye discharges, lethargy, anorexia, and severe spasms of coughing are certain features frequently associated with SIV infection [8]. A hallmark of clinical signs is fever in pigs infected with SIV [8]. The febrile response is thought to be mediated by endogenous mediators, generically called endogenous pyrogens [6]. In the classical model of pathogenesis, induction of fever is mediated by the release of pyrogenic cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and interferons. It has been reported that influenza A virus induced production of TNF-α in human and murine monocytes [16]. TNF-α is a well-known endogenous pyrogen [15]. The present study was undertaken to test the hypothesis that production of TNF-α in the pigs experimentally infected with SIV, is associated with the fever and pulmonary lesion score.

MATERIALS AND METHODS

In vitro infection: Porcine alveolar macrophages were obtained from pigs which were seronegative for porcine circovirus, porcine reproductive and respiratory syndrome virus, and SIV as previously described [3]. Alveolar macrophages were washed with complete medium (RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 30 μg/ml gentamicin), and seeded onto a six well plate at 3 × 10⁶ cells/well. After incubation for 6 hr at 37°C, nonadherent cells were removed and macrophages were infected with SIV and incubated in RPMI medium with 10% swine serum. For each treatment, supernatant was obtained at 0, 3, 6, 9, 12, 24, 36, and 48 hr post-inoculation (hpi) for TNF-α assay. Viral infection of macrophages was monitored by in situ hybridization.

The absence of endotoxin in virus stocks, cell culture supernatants, and media was assessed with the Limulus amebocyte lysate assay (Sigma Chemical Co., St. Louis, MO, U.S.A.). The sensitivity of the assay, with a standard of lipopolysaccharide (LPS) from Escherichia coli O55:B5 (Sigma Chemical Co.) was in the range of 2 to 6 pg/ml.

Experimental Design: Thirty colostrum-deprived pigs aged 3 weeks were randomly allocated in equal numbers to an infected or a control group, having been confirmed as seronegative for SIV infection by an enzyme-linked immunosorbent assay (ELISA; IDEXX Laboratories, Westbrook,
ME, U.S.A.). They were maintained in the stainless steel isolators (two pigs per isolate) and fed a commercial sterile milk substitute. Each of 15 pigs in the infected group was then inoculated intranasally with 3 ml of tissue culture fluid containing SIV (2nd passage; \(2 \times 10^8\) TCID\(_{50}\)/ml). Fifteen pigs in the negative control group were exposed in the same manner to uninfected cell culture supernatants. Three pigs from each group were euthanized at 1, 3, 5, 7 and 10 days post-inoculation (dpi). Tissues were collected from each pig at necropsy and fixed in 10% (v/v) phosphate-buffered formalin for 1-2 days before processing for histopathology, in situ hybridization and immunohistochemistry. Broncho-alveolar lavage (BAL) fluid was collected from the lung of each as previously described [2]. The levels of TNF-\(\alpha\) in the BAL fluid were measured immediately. The middle lobe of the lung from pigs experimentally infected with SIV was used for in situ hybridization for SIV and immunohistochemistry for TNF-\(\alpha\), this lobe having been found to show particularly consistent and intense labelling for SIV [12]. All the methods used were previously approved by the Seoul National University, Institutional Animal Care and Use Committee.

Quantitation of TNF-\(\alpha\): Porcine TNF-\(\alpha\) protein levels in cell culture supernatants and BAL fluid were measured with an enzyme-linked immunosorbent assay (ELISA, Endogen, Woburn, MA, U.S.A.) according to manufacturer’s instructions. Recombinant porcine TNF-\(\alpha\) was used as the standard and the assay sensitivity was 38 pg/ml. ELISA assay was performed in triplicate.

In situ Hybridization for SIV: A 411 base pair (bp) segment of H1 HA gene was used as H1 probe. The forward and reverse primers were 5'-GGGACATGTTACCCAGGAGAT-3' (nucleotides 345 to 365) and 5'-CTGCTTGACTCTCTCACTTTGG-3' (nucleotides 756 to 736), respectively. Reverse transcription-polymerase chain reaction (RT-PCR) for H1 HA gene was carried out as previously described [4, 5].

RT-PCR products of H1 HA gene were purified with a 30-kd cut-off membrane filter. The nucleotide sequences of the purified RT-PCR products were determined by means of BigDye chemistry with the ABI Prism Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequencing was performed on the purified RT-PCR products before they were labelled by random priming with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, IN, U.S.A.) according to the manufacturer’s instructions. In situ hybridization was performed as previously described [12].

Immunohistochemistry for TNF-\(\alpha\): Endogenous alkaline phosphatase was quenched with glacial acetic acid 20% for 2 min at 4°C. Slides were then subjected to the optimal pressure cooking as previously described [17]. All slides were incubated with normal goat serum (Sigma Chemical, St Louis, MO, U.S.A.) in phosphate-buffered saline (PBS) for 30 min at room temperature to saturate nonspecific protein-binding sites. Monoclonal mouse anti-porcine TNF-\(\alpha\) antibody [21] (Pierce Biotechnology Inc., Meridian, IL, U.S.A.) was diluted 1 in 50 in Tween 20 (PBS containing Tween 20, 0.1%). The slides were incubated with the antibody overnight at 4°C in a humid chamber.

After three washes with Tween 20, sections were flooded and incubated for 1 hr at 36°C with biotinylated goat anti-mouse IgG (Dako, Glostrup, Denmark) diluted 1 in 200 in Tween 20. The slides were then washed with Tween 20 before being flooded and incubated for 1 hr at 36°C with streptavidin-alkaline phosphatase conjugate (Roche Molecular Biochemicals, Mannheim, Germany). They were then equilibrated with Tris-buffer (pH 8.2) for 5 min at room temperature. The final reaction was produced by immersing the sections in a solution of red substrate (Boehringer Mannheim, Indianapolis, IN, U.S.A.) for 10 min at room temperature. The sections were lightly counterstained with Mayer’s hematoxylin. Microscopic pulmonary lesion assessment: Three lung sections were examined blindly and given an estimated score of the severity of parameters of pulmonary pathology (i.e. atelectasia, epithelial necrosis, hemorrhages, airway plugging, epithelial hyperplasia, interstitial change, leukocyte infiltration) as previously described [14]. Each individual parameter was given a score ranging from 0 to 3, with 0 indicating no lesion visible and 3 indicating severe lesions. These scores were added to obtain an overall score for each section.

Morphometric analysis: For morphometric analysis of in situ hybridization and immunohistochemistry, three sections were cut from each of three blocks of tissue from the middle pulmonary lobe of each pig. To obtain quantitative data, the morphometric analysis of in situ hybridization slides was performed with the NIH Image J Program (National Institutes of Health, Bethesda, MA, U.S.A.). In each case, 10 fields were randomly selected and the number of positive cells per unit area (0.95 mm²) was counted in respect of bronchial and bronchiolar epithelial cells, pneumocytes, and alveolar and interstitial macrophages. Bronchial and bronchiolar epithelial cells line the bronchus and bronchiole, respectively. Pneumocytes line the interalveolar septa and are flat cells with a centrally placed nucleus. Alveolar macrophages are located in the alveolar space and possess large oval nuclei with abundant cytoplasm. Interstitial macrophages are located in the interstitium and possess large oval nuclei with abundant cytoplasm. The mean values were also calculated.

Statistical analysis: The relationship between 2 parameters was assessed by the Pearson correlation analysis. Wilcoxon matched pairs signed rank test was used to compare infected pigs at each time point with the previous time point in respect of mean number of cells (positive for nucleic acid of SIV or positive for protein of TNF-\(\alpha\)) per unit area of lung and TNF-\(\alpha\) levels in supernatants and BAL fluid. Student’s unpaired t-test was used to compare between infected and uninfected pigs at each time point in respect of mean body temperature and TNF-\(\alpha\) levels in supernatants and BAL fluid. A value of P<0.05 was considered significant.
RESULTS

Concentration of TNF-α in supernatants and BAL fluids: In macrophage culture supernatants, significant differences were not detected in TNF-α concentration between the infected and uninfected group at baseline (0 h) in comparison of the groups at each time point. However, significant differences in TNF-α concentration between SIV-infected and uninfected alveolar macrophages were detected at 3, 6, 9, 12, 24, 36 and 48 hpi. TNF-α concentration was significantly increased at 36 hpi compared to previous time (24 hpi) in infected group (Fig. 1).

TNF-α was not detected in BAL fluids from pigs in the uninfected group. In BAL fluid, therefore, significant differences in TNF-α concentration were detected at 1, 3, 5, 7 and 10 dpi between the infected and uninfected group. TNF-α concentration was maximal at 1 dpi, declined markedly by 3 dpi \((P<0.05)\) and steady thereafter (Fig. 2).

Body temperature: Respiratory disease in the infected group was characterized by the typical sign of influenza. All the infected pigs developed slight pyrexia at 1 dpi. Mean rectal temperatures were above 40°C for pigs in the infected group at 1 dpi, declined markedly by 3 dpi (Fig. 2). Mean rectal temperatures for pigs in the uninfected group were not significantly changed throughout the experiment. Mean rectal temperature for pigs in the infected group was significantly higher than that for pigs in the uninfected group at 1 dpi \((P<0.001)\) (Fig. 2). Body temperatures were correlated with the levels of TNF-α in BAL fluid from pigs experimentally infected with SIV \((r_s=0.9754, P<0.05)\).

In situ Hybridization for swine influenza virus: Positive cells typically exhibited a dark brown reaction product in the nucleus and cytoplasm without any background staining. Strong hybridization signals were detected mainly in the bronchial and bronchiolar epithelial cells (Fig. 3a) at 1 and 3 dpi, and mainly in the pneumocytes and macrophages (alveolar and interstitial) at 7 and 10 dpi. Cells positive for SIV was significantly decreased at 3 and 5 dpi \((P<0.05)\). No hybridization signal was seen in the tissue sections pre-treated with RNase A. Sections from the 15 control pigs showed no hybridization signal for SIV.

Immunohistochemistry for TNF-α: Positive cells typically exhibited a red reaction product without any background staining. The distribution of TNF-α antigen depended on the duration of infection. Thus, hybridization signals were detected mainly in the bronchial and bronchiolar epithelial cells at 1 and 3 dpi, and mainly in the pneumocytes and macrophages (alveolar and interstitial) at 7 and 10 dpi. There was cell-to-cell correlation when serial sections were examined by in situ hybridization of SIV and immunohistochemistry of TNF-α (Fig. 3a, b, c, d). Sections from the 15 control pigs showed no immunohistochemical signal for TNF-α.

The number of positive cells for TNF-α was significantly decreased at 3 and 5 dpi (Fig. 4). Rank was positively correlated between TNF-α and SIV \((r_s=0.9629, P<0.001)\). The pulmonary lesion scores were correlated with the means of positive cells by immunohistochemistry for TNF-α \((r_s=0.9949, P<0.001)\) (Fig. 5).

DISCUSSION

The results of this study indicate the production of TNF-α in pigs experimentally infected with SIV and a possible contribution of this cytokine to the major clinical manifestation of the disease. Intense and consistent expression of TNF-α antigen was demonstrated in the pulmonary lesions caused by SIV. In contrast, the expression of cytokine mRNA was minimal in non-lesional lungs of the infected pigs and in normal lungs from the control pigs. Serial sections of the lungs indicated that areas containing numerous
TNF-α-positive cells also have numerous SIV-positive cells in this study. Thus, in vitro and in vivo study clearly showed that the protein expression of TNF-α may be a direct effect of SIV. Moreover, the distribution of swine influenza and TNF-α antigen varied with the duration of infection. The simultaneous detection of SIV and TNF-α was observed in the bronchial and bronchiolar epithelial cells in early infection, but in the alveolar and interstitial macrophages in late infection. These cells are the main source of TNF-α production [1, 16]. TNF-α-positive cells were detected in the lung at 1 dpi, and gradually decreasing thereafter. The results strongly suggest that TNF-α is an important mediator in the pathophysiology of SIV infection.

A sharp increase in TNF-α concentration was seen in macrophages by early infection of SIV, indicating that the kinetics of TNF-α production is rapid. Our in vivo data also show that the participation of TNF-α occur in the acute phase of infection, with the maximal fever by 1 dpi. Macrophages, and the bronchial and bronchiolar epithelia are the target cells for SIV [11, 12] and this infection causes TNF-α secretion, which is linked to a variety of disease symptoms. TNF-α is a known endogenous pyrogen [16]. Fever is a clinical sign observed in pigs infected with SIV [8]. It seems, therefore, that TNF-α secreted by macrophages contributes to the fever observed during early SIV infection. The present study provides the direct evidence that TNF-α is elevated in BAL fluid and selectively expressed at the site of pulmonary lesions, providing further support for the role of this cytokine in pathogenesis of SIV infection.

An influenza A virus infection alone induced a marked accumulation of TNF-α mRNA which was only minimally translated into bioactive TNF-α protein in humans [16]. A large amount of TNF-α production was induced when a secondary stimulus of LPS was added [9, 16]. SIV infection is often seen in combination with other bacterial pathogens (Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Haemophilus spp., and Pasteurella multocida) in pigs [7, 8]. Data from this study indicate that a correlation exists between the level of TNF-α and pulmonary lesion score; hence, the more production of TNF-α induces the more severe lesions. In the field study, a combination of these bacterial pathogens with SIV often produces more protracted respiratory disease, higher mortality than is seen in uncomplicated SIV infection [8]. Therefore, it is speculated that such an enormous TNF-α release from macrophages might be partially responsible for the serious clinical complication of combined swine influenza A virus and bacterial infections.
TNF-α plays an important role in the induction and regulation of inflammatory responses. TNF-α also showed strong antiviral activity against avian, swine and human influenza virus, and the antiviral effect of TNF-α was greater than that of IFN-α and -γ [20]. Moreover, together with IFN-γ, it leads to the activation of macrophages, improving their ability to direct antiviral effects. The previous study showed that the production of IFN-γ was increased at 5 dpi with maximal production at 7 dpi in BAL fluid from pigs experimentally infected with SIV subtype H1N1 [10]. These data strongly indicated that TNF-α may act synergistically with IFN-γ, inhibiting the replication of SIV in the late phase of infection like other several RNA viruses [22]. Further studies are needed to define the antiviral role of TNF-α in the late phase of SIV infection.

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REFERENCES


