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SHORT COMMUNICATION

Expression of myeloperoxidase in swine influenza virus (SIV)-infected neutrophils in lungs from pigs experimentally infected with SIV subtype H1N2

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Abstract The expression of myeloperoxidase (MPO) was examined in the swine influenza virus (SIV)-infected neutrophils in the lungs of pigs experimentally infected with swine influenza virus (SIV) subtype H1N2 by immunohistochemistry. Five pigs each from the infected and non-infected group were euthanized 1, 3, 5, 7, and 10 days post-inoculation (dpi). Immunohistochemical reactivity was mainly seen in neutrophils. The score for pulmonary histopathological lesions correlated with the score for MPO immunohistochemical reactivity (r_s=0.962, P<0.01). In addition, the score for in situ hybridization of SIV nucleic acid correlated with the score for MPO immunohistochemical reactivity (r_s=0.976, P<0.01). These results suggest neutrophils are one of the primary effector cells in the early phase of SIV infection in pigs.

Keywords Neutrophil · Myeloperoxidase · Pig · Swine influenza virus

Bongtae Kim and Jeoung Hwa Shin made an equal contribution to this work, they are co-first authors

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Introduction

Innate (non-specific) and adaptive (specific) immunity are important host defenses against influenza virus (Tate et al. 2008). Although the development of adaptive immunity is important in the recovery from influenza virus infection, innate defense mechanisms may be more important because the nature of disease progresses acutely before a specific immune response develops. Neutrophils are important host defense cells against influenza virus during the phase of innate immunity. Although the role of neutrophils against influenza virus infections has been debated, neutrophils have been shown to play a role in the control and clearance of influenza virus in experimental models (Fujisawa 2008; Salvatore et al. 2007; Tate et al. 2008; Tecle et al. 2007; White et al. 2007). It has been reported that the influenza virus was inactivated by myeloperoxidase (MPO) from human neutrophils in the presence of H_2O_2 (Yamamoto et al. 1991). This suggests a role of MPO in neutrophils for the pathophysiology of influenza virus infection. The present study was undertaken to determine if expression of MPO in neutrophils is associated with number of viral infected cells and pulmonary lesion score in the lungs from pigs experimentally infected with SIV.

Materials and methods

Animals

Fifty colostrum-deprived pigs, aged 3 weeks, were randomly allocated in equal numbers to an infected or a control group. All pigs were confirmed to be seronegative for SIV infection by an enzyme-linked immunosorbent assay (ELISA; IDEXX Laboratories, Westbrook, ME, USA). The pigs were maintained in stainless steel isolators (two pigs per isolator) and fed a commercial sterile milk substitute.

Experimental design

Swine influenza virus H1N2, A/SW/Kyounggi/0981/03 (SNUVR030925 strain), was used in this study (Jung and Chae 2004). Each of the 25 pigs in the infected group was then inoculated intranasally with 3 ml of tissue culture fluid containing SIV (2nd passage; 2×10^6 $TCID_{50}$/ml) while, each of the 25 pigs in the uninfected group was inoculated intranasally with 3 ml of tissue culture fluid. Five pigs from each group were euthanized 1, 3, 5, 7, and 10 days post-inoculation (dpi). Tissues were collected from each pig at necropsy. The middle lobe of the lung from pigs experimentally infected with SIV was used for in situ hybridization for SIV and immunohistochemistry for MPO, as this lobe was found to show particularly consistent and intense labeling for SIV (Jung et al. 2005). All the methods used were previously approved by the Seoul National University Institutional Animal Care and Use Committee.

In situ hybridization

A 411 base pair (bp) segment of H1 HA genes was used as H1 probe. The forward and reverse primers were 5'-GGGACATGTTACCCAGGAGAT-3' (nucleotides 345 to 365) and 5'-CTGCTTGACCTCTCACTTTGG-3' (nucleotides 756 to 736), respectively. 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, USA). Sequencing was performed on the purified RT-PCR products before they were labelled by random priming with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's instructions. In situ hybridization was performed as previously described (Jung et al. 2005).

Immunohistochemistry

Endogenous alkaline phosphatase was quenched with glacial acetic acid 20% for 2 min at 4°C. Slides were then subjected to optimal pressure cooking as previously described (Norton et al. 1994). All slides were incubated with normal goat serum (Sigma Chemical, St Louis, MO, USA) in phosphate-buffered saline (PBS) for 30 min at room temperature to saturate nonspecific protein-binding sites. Polyclonal rabbit anti-human MPO antibody (Dako, Glostrup, Denmark) was diluted 1 in 1000 in Tween 20 (PBS containing Tween 20, 0.1%). The slides were incubated with antibody overnight at 4°C in a humid chamber. MPO antibodies used in this study were cross-reactive to porcine myeloperoxidase (Pinkus and Pinkus 1991).

After three washes with Tween 20, sections were flooded and incubated for 1 h at 36°C with biotinylated goat anti-rabbit 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 h 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 (Fast red, Roche Diagnostic GmbH, Mannheim, Germany) for 10 min at room temperature. The sections were lightly counterstained with Mayer's hematoxylin.

Morphometric analysis

Microscopic pulmonary lesion scores were assessed as previously described (Jung et al. 2005). For morphometric analysis of in situ hybridization and immunohistochemisty, three sections were cut from each of three blocks of tissue from the right and left middle pulmonary lobe of each pig and assessed as previously described (Jung et al. 2005).

Statistical analysis

The relationship between two parameters was assessed by the Pearson correlation analysis. The Wilcoxon matched pairs signed rank test was used to compare in infected pigs each time point with the previous time point in respect of mean number of cells (positive for nucleic acid of swine influenza virus or positive for protein of MPO) per unit area of lung. A value of $P<0.05$ was considered significant.

Results

Microscopic lesions were observed in the lungs of all infected pigs. The lesions were multifocal in distribution and generally were associated with bronchioles. They consisted of epithelial cell damage, airway plugging, and peribronchial and peribron-

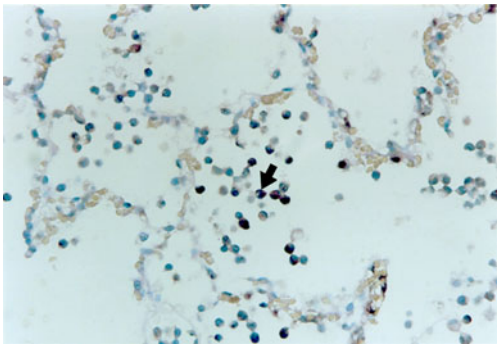

Fig. 1 Lung from a pig experimentally infected with swine influenza virus (SIV) subtype H1N2 at 1 dpi. SIV nucleic acid (dark brown reaction) was detected in neutrophils with bilobed nuclei (arrow) by in situ hybridization

chiolar infiltration by inflammatory cells. Pneumonia was severe at 1 dpi, moderate at 3 and 5 dpi, and mild at 7 and 10 dpi.

The 25 infected pigs showed distinct positive hybridization signals. Positive cells typically exhibited a dark brown reaction product in the nucleus and cytoplasm without any background staining. When lung tissues from these pigs were hybridized with the nonradioactive digoxigenin-labeled cDNA probe, a strong hybridization signals were detected mainly in the bronchial and bronchiolar epithelial cells, and neutrophils (Fig. 1) at 1 and 3 dpi but mainly in the pneumocytes and macrophages (alveolar and interstitial) at 7 and 10 dpi. No hybridization signal was seen in tissue sections pretreated with RNase A. The cDNA probes for PRRSV were consistently negative in all tissue tested. Sections from the 25 control pigs showed no hybridization signal for SIV.

The 25 infected pigs showed distinct positive immunohistochemical signals for MPO. Positive cells typically exhibited a red reaction product in the cytoplasm without any background staining. Immunohistochemical reactivity was mainly seen in neutrophils, which had bilobed or multilobed nuclei (Fig. 2). The immunohistochemical signal was rarely seen in macrophages, which had large oval nuclei and abundant cytoplasm. The immunohistochemical signals for MPO antigen were confined to neutrophils mainly in area that had hybridization signal for SIV (Fig. 3a and b). Sections from the 25 control pigs showed no immunohistochemical signal for MPO.

Statistical analysis of the mean number of pulmonary histopathological lesions, SIV-positive cells, and MPO-positive cells per unit area of lung indicated there was a significant difference according to the duration of infection. The score for pulmonary histopathological lesions

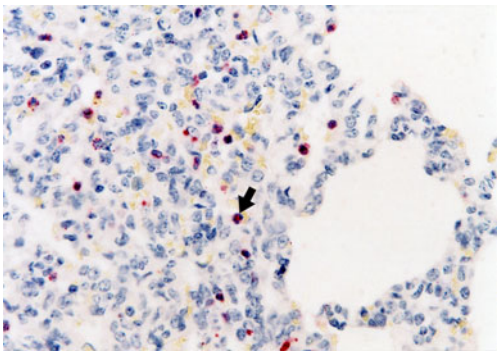

Fig. 2 Lung from a pig experimentally infected with swine influenza virus (SIV) subtype H1N2 at 1 dpi. Myeloperoxidase protein was detected in neutrophils with bilobed nuclei (arrow) by immunohistochemistry

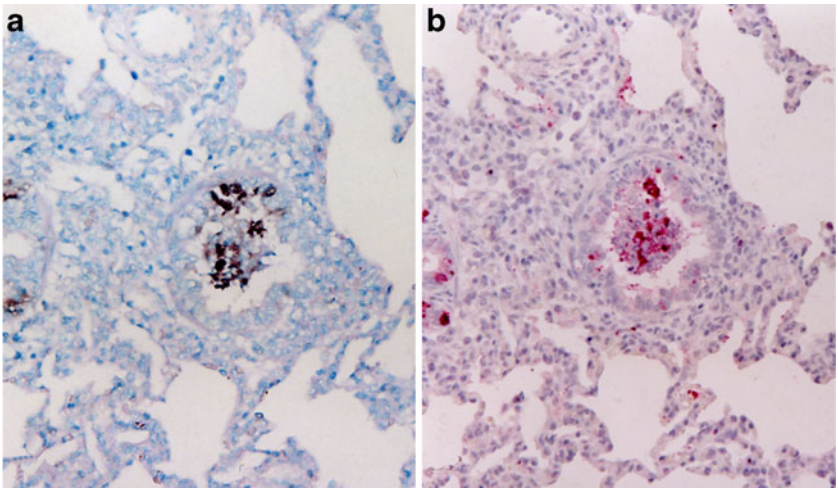

Fig. 3 Lung from a pig experimentally infected with swine influenza virus (SIV) subtype H1N2 at 1 dpi. SIV nucleic acid (dark brown reaction) was detected in mainly neutrophils in the lumen of bronchioles by in situ hybridization (**a**). Serial sections showing that contiguous cells were positive for myeloperoxidase by immunohistochemistry (**b**)

decreased significantly between 1 and 3 dpi ($P<0.05$). From 1 to 5 dpi, significantly fewer SIV-positive cells were detected in the lungs ($P<0.05$). The score for MPO-positive cells decreased from 1 to 10 dpi ($P<0.05$). The score for pulmonary histopathological lesions correlated with the score for MPO immunohistochemical reactivity ($r_s=0.962$, $P<0.01$). In addition, the score for in situ hybridization of SIV nucleic acid correlated with the score for MPO immunohistochemical reactivity ($r_s=0.976$, $P<0.01$) (Fig. 4).

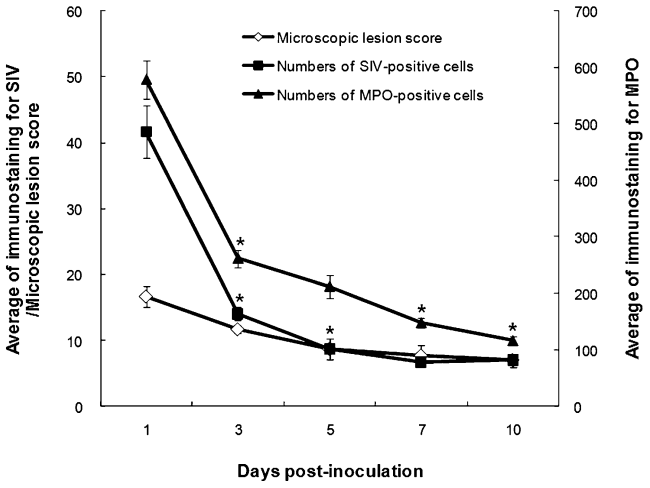

Fig. 4 Mean immunohistochemistry score for myeloperoxidase, mean score for in situ hybridization of swine influenza virus (SIV) subtype H1N2, and mean score for pulmonary histopathological lesions in the lungs from pigs experimentally infected with SIV subtype H1N2. (*) indicates a statistically significant difference relative to the previous time point ($P<0.05$)

Discussion

The results of this study demonstrated SIV nucleic acid was present in the neutrophils by in situ hybridization. Positive signals for SIV nucleic acid were observed in the neutrophils by in situ hybridization yet, it was not clear whether the SIV virus can replicate in the porcine neutrophil although the human influenza virus can replicate in human neutrophils (Zhao et al. 2008). One important function of neutrophils is phagocytosis. Hence, the SIV detected in the neutrophils may have resulted from uptake. In vitro, human neutrophils preferentially adsorb influenza viruses and bound viruses are ingested and digested in phagosomes (Yamamoto et al. 1989). Further study needs to determine whether SIV can replicate in the porcine neutrophils.

In situ hybridization and immunohistochemistry of serial sections of lung indicated that areas containing SIV-positive cells also had MPO-positive cells. These observations suggested that SIV may induce the expression of MPO in neutrophils. However, functional role of MPO is not well clarified at this time. In the human study, expression of MPO may be linked with the antiviral activity. MPO inactivated hemagglutination activity of human influenza virus, which is a key factor in the initiation of human influenza virus infection in human host cells (Choppin and Scheid 1980). In addition, MPO modified viral major proteins, including the inner proteins of the envelope and seems to cause alteration of the protein configuration essential for viral infectivity in human influenza virus (Yamamoto et al. 1991). Instead of antiviral activity, MPO released from neutrophils which are activated by the avian H5N1 virus could damage lung tissues in human patients with acute respiratory distress syndrome (Liem et al. 2008). Moreover, increased MPO activity in plasma of avian H5N1-positive human patients plays key roles in acute lung injury (Phung et al. 2011). Although MPO expression is correlated with the pulmonary histopathological lesion in the present study, further study is needed to determine the functional role of MPO in neutrophils to limit the replication of SIV in lungs or induce acute lung injury.

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References

Choppin PW, Scheid A (1980) The role of viral glycoproteins in adsorption, penetration, and pathogenicity of viruses. Rev Infect Dis 2:40–61

Fujisawa H (2008) Neutrophils play an essential role in cooperation with antibody in both protection against and recovery from pulmonary infection with influenza virus in mice. J Virol 82:2772–2783

Jung K, Chae C (2004) Phylogenetic analysis of an H1N2 influenza A virus isolated from a pig in Korea. Arch Virol 149:1415–1422

Jung K, Ha Y, Chae C (2005) Pathogenesis of swine influenza virus subtype H1N2 infection in pigs. J Comp Pathol 132:179–184

Liem NT, Nakajima N, Phat LP, Sato Y, Thach HN, Hung PV, San LT, Katano H, Kumasaka T, Oka T, Kawachi S, Matsuchita T, Sata T, Kudo K, Suzuki K (2008) H5N1-infected cells in lung with diffuse alveolar damage in exudative phase from a fatal case in Vietnam. Jpn J Infect Dis 61:157–160

Norton AJ, Jordan S, Yeomans P (1994) Brief, high-temperature heat denaturation (pressure cooking) - a simple and effective method of antigen retrieval for routinely processed tissues. J Pathol 173:371–379

Phung TTB, Luong ST, Kawachi S, Nunoi H, Nguyen LT, Nakayama T, Suzuki K (2011) Interleukin 12 and myeloperoxidase (MPO) in Vietnamese children with acute respiratory distress syndrome due to avain influenza (H5N1) infection. J Infect 62:104–108

Pinkus GS, Pinkus JL (1991) Myeloperoxidase - a specific marker for myeloid cells in paraffin sections. Mod Pathol 4:733–741

Salvatore M, Garcia-Sastre A, Ruchala P, Lehrer RI, Chang T, Klotman ME (2007) Alpha-defensin inhibits influenza virus replication by cell-mediated mechanism(s). J Infect Dis 196:835–843

Tate MD, Brooks AG, Reading PC (2008) The role of neutrophils in the upper and lower respiratory tract during influenza virus infection of mice. Respir Res 9:57

Tecle T, White MR, Gantz D, Crouch EC, Hartshorn KL (2007) Human neutrophil defensins increase neutrophil uptake of influenza A virus and bacteria and modify virus-induced respiratory burst responses. J Immunol 178:8046–8052

White MR, Tecle T, Crouch EC, Hartshorn KL (2007) Impact of neutrophils on antiviral activity of human bronchoalveolarlavage fluid. Am J Physiol Lung Cell Mol Physiol 293:L1293–L1299

Yamamoto K, Suzuki K, Suzuki K, Mizuno S (1989) Phagocytosis and ingestion of influenza-virus by human polymorphonuclear leukocytes *in vitro*: electronmicroscopy studies. J Med Microbiol 28:191–198

Yamamoto K, Miyoshikoshio T, Utsuki Y, Mizuno S, Suzuki K (1991) Virucidal activity and viral protein modification by myeloperoxidase - a candidate for defense factor of human polymorphonuclear leukocytes against influenza-virus infection. J Infect Dis 164:8–14

Zhao YY, Lu M, Lau LT, Lu J, Gao ZF, Liu JH, Yu ACH, Cao Q, Ye JX, McNutt MA, Gu J (2008) Neutrophils may be a vehicle for viral replication and dissemination in human H5N1 avian influenza. Clin Infect Dis 47:1575–1578