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Influenza A virus PB1-F2 is involved in regulation of cellular redox state in alveolar epithelial cells

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ABSTRACT

Occurrence of oxidative stress is common in influenza, and renders the host more susceptible to pathogenic effects including cell death. We previously reported that down-regulation of superoxide anion dismutase 1 (SOD1) by influenza A virus (IAV) resulted in a significant increase in the levels of reactive oxygen species (ROS) and viral PB1 polymerase gene product in the early stage of infection. However, the precise molecular mechanism of IAV-mediated ROS generation is not yet fully understood. In this study, we investigated the possible involvement of the key virulence factor PB1-F2 in ROS generation and its contribution to the viral propagation and cell death. The key virulence factor PB1-F2 was found to be responsible, at least in part, for the ROS generation through lowering the SOD1 level in alveolar epithelial A549 cells. PB1-F2 overexpression resulted in SOD1 diminishment and ROS enhancement, while another virulent factor, NS1, did not show significant changes. Inversely, we examined the effects of the absence of PB1-F2 using mutant IAV lacking PB1-F2 expression (mutantΔF2). Infection with mutantΔF2 virus did not significantly lower the SOD1 level, and thus generated moderately low levels of ROS. In addition, the oxidative activity of PB1-F2 was directly reflected by cell viability and death. Infection with the mutant virus reduced the percentage of apoptotic cells more than two-fold compared to the wild-type IAV in A549 cells. Furthermore, expression of exogenous SOD1 gene abrogated a large portion of the PB1-F2-induced apoptosis of cells infected with wild-type IAV, but affected much less of the mutantΔF2 virus-infected cells. These results suggest that the PB1-F2 is directly implicated in virus-induced oxidative stress, thereby contributing to the early stages of IAV replication cycle and ultimately to disease severity.

1. Introduction

During infection with viruses including influenza A virus (IAV), reactive oxygen species (ROS) have often emerged due to disturbance of redox balance [1]. The oxidative stress generated by imbalance of cellular redox state induces a variety of adverse effects, including DNA damage, inflammation and dysregulation of cell proliferation and growth [2–4]. Therefore, IAV-induced ROS may play an important role in the pathogenicity of IAV, a major human and animal respiratory pathogen. A number of viruses are known to be often accompanied by excess ROS during infection, which contributes to enhanced disease pathogenicity [5–7]. Many reports have demonstrated that IAV causes the generation of excess ROS, and the IAV-mediated oxidative stress was implicated in pathogenesis in respiratory cells and tissues [8,9]. However, a variety of cellular mechanisms are utilized to cope with the ROS generation and to maintain a normal cellular redox state during virus infection. Among the many cellular factors which defend against ROS generation, members of the superoxide dismutase (SOD) family are ubiquitous components of cellular detoxification systems, as the major cellular antioxidant enzymes. In the previous study, it was shown that IAV infection induced a significant decrease in the SOD1 level, which consequently allowed increase of ROS generation [10]. IAV infection caused the specific down-regulation of Sp1 at both the transcriptional and post-translational levels, thereby leading to the diminishment of Sp1-dependent SOD1 expression immediately after IAV infection. This evidence supports that prolonged exposure to excess ROS after infection is implicated in enhanced tissue damage; however, the

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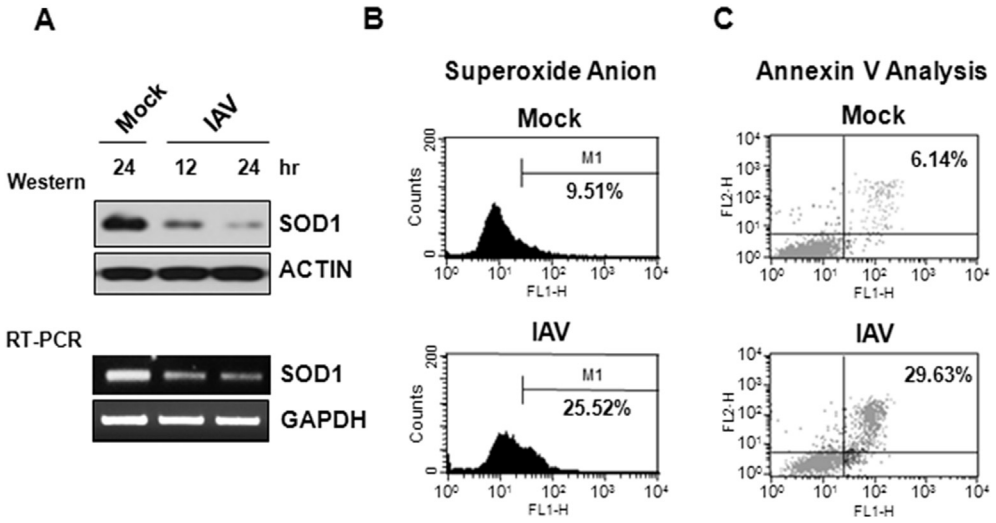

Fig. 1. The critical role of IAV-induced SOD1 in ROS generation and host cell death. (A) Human lung alveolar epithelial A549 cells were infected with IAV at an MOI of 0.01 for the indicated time period. Infected cells were harvested and total proteins were analyzed by Western blot for SOD1 and actin (upper). Total RNA was subjected to RT-PCR for the indicated genes (lower). (B) A549 cells were infected with IAV at an MOI of 0.01. At 24 h of post-infection (hpi), cells were stained with superoxide anion sensitive dye and analyzed using a flow cytometer. (C) Representative FACS showing percentage of apoptotic cells following IAV infection of A549 cells.

mechanisms by which IAV causes an increase in ROS generation and viral infectivity are not yet well understood.

Some IAV proteins have been involved in the host defense to IAV infection. A multifunctional protein of IAV, NS1, was extensively studied and identified to have a role in suppression of host antiviral response via induction of IFN-α/β [11]. The 11th IAV gene product, PB1-F2, is encoded in the +1 reading frame of the IAV polymerase PB1 gene segment [4]. PB1-F2 was identified to interact with the mitochondrial permeability transition pore complex components, and might thus play a role in the induction of mitochondria-associated apoptosis and tissue damage [12,13]. However, recent reports suggest that the function of PB1-F2 in IAV pathogenicity seems to be cell type and virus strain specific [14,15]. Therefore, to date, the function of the PB1-F2 protein has not yet been clearly elucidated. In the present study, we attempted to identify the role of PB1-F2 in IAV (A/WSN/1933)-induced oxidative stress and its consequent effects on host cell death as well as viral gene expression, tested in the presence or absence of PB1-F2 expression, in alveolar epithelial A549 cells.

2. Materials and methods

2.1. Cell culture

Human alveolar epithelial cell A549 cells and Madin–Darby canine kidney (MDCK) cells were purchased from ATCC (Rockville, MD, USA). These cells were maintained and cultured as described previously [16].

2.2. Production of virus

Eight segmented-single fragmented genes of IAV (A/WSN/1933) were used for generation of the recombinant influenza virus as described previously [10]. The eight plasmids were generously provided by R.G. Webster (University of Tennessee, TN, USA). The virus titer was determined on MDCK cells by plaque assays as described previously [17].

2.3. Plasmids

A mutant form of A/WSN/1933 IAV lacking PB1-F2 expression, mutant△F2, was constructed by replacing the start codon (T to C at position 95) of the PB1-F2 open reading frame (ORF) of the PB1 segment. In addition to the stat codon replacement, an additional stop codon was generated at amino acid 12 of PB1-F2 (C to G at position 129) to ensure complete disruption of PB1-F2 expression. The amino acid replacements did not modify the PB1 ORF. Flag-tagged protein expression plasmids, pFlag-F2 and pFlag-NS1, were also generated. The ORFs of PB1-F2 and NS1, originating from the pHW182-PB1-F2 and pHW182-NS1 segments, were amplified by PCR, and subcloned into the p3xFLAG-CMV10 backbone vector to yield pFlag-F2 and pFlag-NS1, respectively. Two separate sets of primers were used for each fragment. The primer set for the PB1-F2 was (5′-CGAATTCCATGGGACAGGAACAGGATAC-3′ and 5′-CTCTAGATCAGCTTGTCCACTCGTGTT-3′); the primer set for the NS1 was (5′-CGAATTCCATGGATCCAAACACTGTGTC-3′ and 5′-CTCTAGATTAAATAAGCTGAAACGAG-3′).

2.4. Measurement of intracellular superoxide anion

Transfected or infected cells were seeded at 2×10^5 cells in 6 well plates. The cells were washed with PBS, treated with 0.4% trypsin and collected. The level of superoxide anion was analyzed using the superoxide detection system (Enzo Life Sci.) following the manufacturer's instructions. The cells were incubated with superoxide staining solution for 30 min at 37 °C, and immediately analyzed using a flow cytometer (FACS Caliber, Becton and Dickinson).

2.5. Reverse transcription (RT)-PCR

Total RNAs were extracted from cells using TRIzol reagent (Invitrogen). RT-PCR was done using SuperScript III reverse transcriptase according to manufacturer's instructions (Invitrogen). The cDNAs were PCR-amplified by primers specific for the indicated genes. Each primer is as follows. SOD1 (forward), 5′-ACAAAGATGGTGTGGCCGAT-3′; SOD1 (reverse), 5′-TGGGCGATCCCAATTACACC-3′; PB1-F2 (forward), 5′-CGAATTCCATGGGACAGGAACAGGATAC-3′; PB1-F2 (reverse), 5′-CTCTAGATCAGCTTGTCCACTCGTGTT-3′;

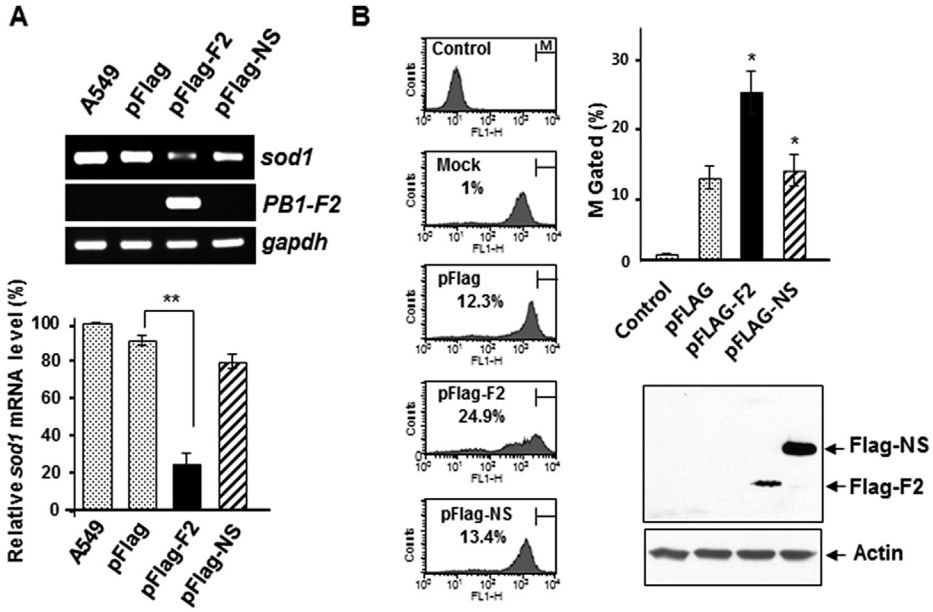

Fig. 2. PB1-F2 expression influences levels of SOD1 and ROS in A549 cells. (A) A549 cells were transfected with the indicated plasmid. Transfected cells were harvested and total RNA was analyzed by RT-PCR with primers specific for the indicated genes. A representative RT-PCR analysis was shown (upper). Quantitative intensity of the bands was determined using densitometry (lower). Image J program (NIH, MD, USA) was used to calculate each relative unit of SOD1 mRNA level after normalization with GAPDH mRNA level. (B) Detection of endogenous levels of superoxide anion. Prior to staining, cells were transfected with the indicated plasmid. After 48 h, cells were treated with the superoxide detection system (Enzo Life Sci.) and then examined using flow cytometry (left). Percentages of the superoxide positive population are indicated. The graph (top right) represents the level of superoxide anion in the transfected cells. Western analysis (bottom right) confirmed the expression of PB1-F2 (Flag-F2) and NS1 (Flag-NS) after transfection. Data are means ± SD of at least three separate experiments. Statistical significance was relative to the cells transfected with pFlag, and was determined using the *t*-test (*$P < 0.05$, **$P < 0.01$).

GAPDH (forward), 5′-AGAAGGCTGGGGCTCATTTG-3′; GAPDH (reverse), 5′-AGGGGCCATCCACAGTCTTC-3′. PCR products were separated on an agarose gel and stained with ethidium bromide. Quantitative intensity of the bands was determined using densitometry. The densitometry data for band intensities was generated by analyzing the gel images on the Image J program (Version 1.33, NIH Image, Bethesda, MD, USA) and normalized to the amount of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) transcripts [18].

2.6. Western blot analysis

All procedures for Western blot were carried out as previously described [19]. Antibodies against SOD1 and actin were purchased from Sigma Aldrich (St. Louis, MO., USA). Antibody against NP was purchased from GeneTex (Irvine, CA., USA). Antibody against PB1-F2 was generously provided by P. Palese (Mount Sinai School of Medicine, NY, USA).

2.7. Annexin V and WST-1 assay

2×10^5 cells were seeded in 6-well plates and then infected with influenza A virus for 1 h. The cells were harvested after incubation for 24 h. Analysis was then carried out using flow cytometry (FACS Caliber, Becton and Dickinson) with FITC-labeled annexin V antibody. To assay cell proliferation, cells were incubated with water-soluble tetrazolium salt-1 (WST-1) reagent (Roche, Indianapolis, IN, USA) at 37 °C for 5–10 min. A microplate spectrophotometer was used to measure the absorbance of the samples at 450 nm.

2.8. Immunofluorescence analysis

After infection of A549 cells with the virus, SOD1 and NP proteins were detected by fluorescence microscopy. Immunofluorescence assay was carried out as described elsewhere [20]. Images were analyzed using a Zeiss Axioskop 2 microscope configured for epifluorescence.

3. Results

3.1. IAV-induced SOD1 regulation plays a critical role in ROS generation and host cell death

As IAV-mediated oxidative stress appears to be involved in pathogenesis in respiratory cells, it was investigated whether IAV infection alters the level of SOD1 and ROS, and whether such change in ROS can in turn induce host apoptosis. To address this, alveolar epithelial A549 cells were infected with IAV (A/WSN/1933) at an MOI of 0.01, and the expression of SOD1 and generation of superoxide anion were examined. As the number of hour post-infection (hpi) increased, the levels of SOD1 mRNA and protein were decreased (Fig. 1A). At 24 hpi, the cells were analyzed by flow cytometry for generation of ROS. Fig. 1B demonstrated that the enhanced level of superoxide anion in the infected cells could be a reflection of the decreased SOD1 level. At the same hpi, cells were collected and the degree of apoptosis was analyzed using flow cytometry. As shown in Fig. 1C, it is likely that the ROS generated by viral infection appreciably induced apoptosis. These results indicated that the IAV-mediated ROS generation appears to be correlated with induction of apoptosis, although there is no direct information linking each result.

3.2. PB1-F2 expression is closely implicated in SOD1 reduction and ROS generation in A549 cells

In order to determine whether the apoptosis induced by ROS could be attributed to IAV infection, we attempted to identify the viral gene responsible for ROS generation after infection. In

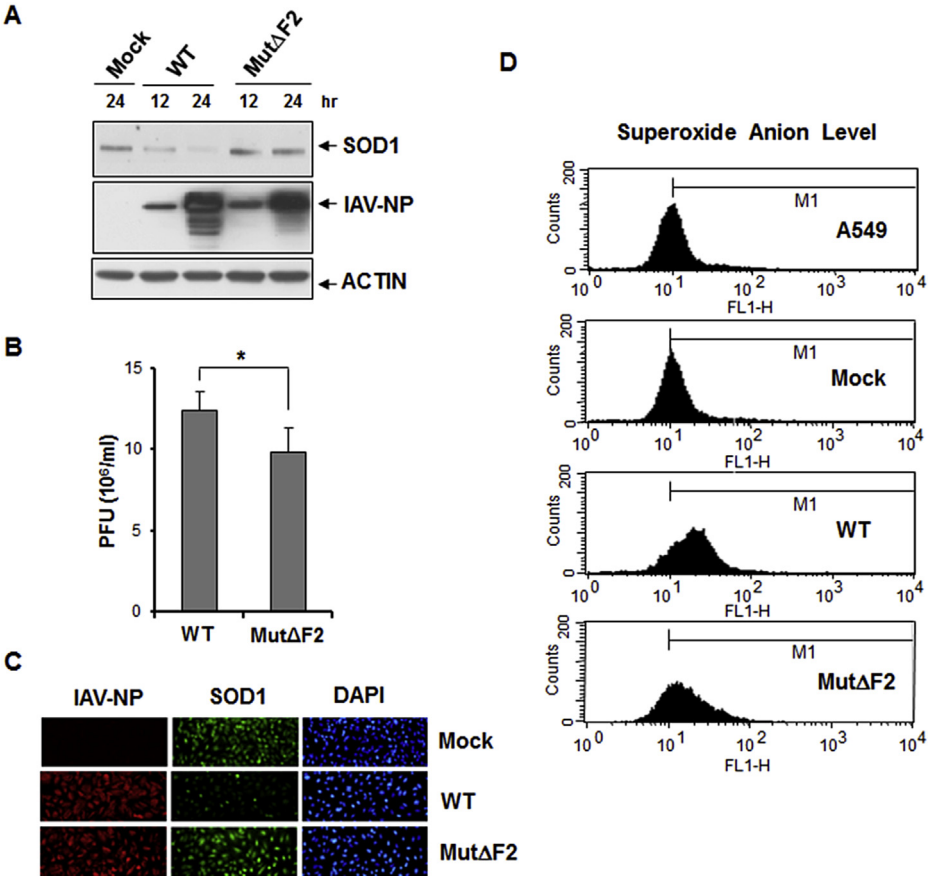

Fig. 3. Influence of IAV mutant lacking PB1-F2 expression on cellular redox state. A549 cells were infected with either wild-type (WT) or mutant (Mut△F2) virus at an MOI of 0.01. (A) Western blot. After infection for the indicated time period, cells were harvested and total proteins were analyzed with the indicated antibodies. (B) Plaque assay. At 24 hpi, supernatants of infected cells were analyzed for the virus titers of the WT and Mut△F2 as described in the Materials and Methods. $^{*}P < 0.05$. (C) Immunofluorescence analysis. Cells were infected with the indicated virus for 24 h, and visualized with an inverse fluorescence microscope after staining with the antibody against IAV-NP, SOD1, and DAPI. (D) After infection with the indicated virus, cells were incubated for 24 h then treated with superoxide detection system (Enzo Life Sci.), after which flow cytometry was carried out.

preliminary studies, transient transfections of each viral gene segment were carried out in A549 cells, and the mRNA and protein levels of SOD1 antioxidant were analyzed (not shown). Among several viral gene segments, two virulence gene segments were identified to be correlated with the IAV-induced SOD1 down-regulation: PB1-F2 and NS1. For more detailed monitoring, the PB1-F2 and NS1 genes were subcloned into the pFlag vector, yielding pFag-F2 and pFlag-NS, respectively. As shown in Fig. 2A, transfection with pFlag-F2 caused a marked decrease in the SOD1 mRNA level, while transfection with pFlag-NS showed only a slight decrease. The expression of PB1-F2 also enhanced the generation of superoxide anion, whereas the expression of NS1 showed a minor change (Fig. 2B). Therefore, these results support that IAV infection indeed down-regulates *sod1* and enhances ROS level, and that the virulence factor PB1-F2 is responsible for the induction of such host responses.

3.3. Mutant IAV lacking PB1-F2 expression is less active in altering cellular redox state

Next, the effects of PB1-F2 deficiency were determined to investigate its role in IAV-induced pathogenesis. To accomplish this, a PB1 gene segment lacking PB1-F2 expression was generated without changing the open reading frame of PB1, and was subsequently utilized to produce mutantΔF2 virus. The level of SOD1 in response to infection with wild-type and mutantΔF2 virus was then assessed. Western blot analysis showed that the level of SOD1 protein was decreased in the cells infected with wild-type virus, but no significant change occurred in the cells infected with mutantΔF2 virus for 24 h (Fig. 3A). In addition, we observed that viral NP expression slightly decreased when mutantΔF2 virus was infected. Consistent with the NP expression level, we also observed slight differences of progeny virus titers between the recombinant wild type and the PB1-F2 knockout mutant upon infection of A549 (Fig. 3B). This indicates that SOD1 regulation may be correlated with the replication efficiency of IAV. Furthermore, as the turnover rate of SOD1 protein is so high in the IAV-infected cells, immunofluorescence analysis was performed for quantitative comparison of the difference in SOD1 levels in the intact cells. As expected, the immunofluorescence for SOD1 was substantially lower in wild-type virus-infected cells than in cells infected with mutantΔF2 virus (Fig. 3C). We also observed that the level of NP was slightly reduced in cells during the infection with mutantΔF2 virus although it was not significant compared to the change in the level of SOD1, indicating the potential of PB1-F2 to contribute, at least in part, to viral propagation.

In addition, the level of superoxide anion was determined after infection with either wild-type or mutantΔF2 virus in order to investigate whether PB1-F2-mediated SOD1 downregulation is responsible for the generation of superoxide anion. Whereas wild-type virus led to considerable enhancement of the level of superoxide anion, the mutant virus showed a minor change

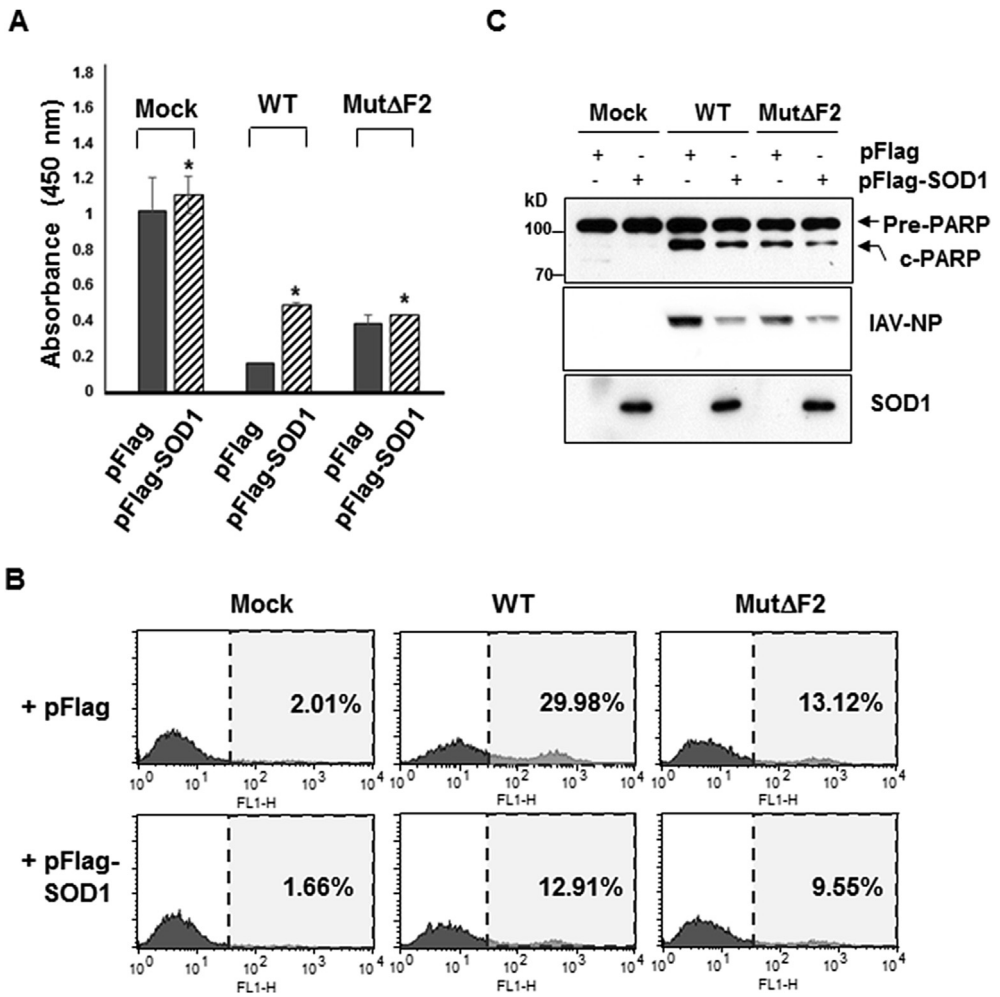

Fig. 4. Rescue of PB1-F2-mediated cell death by exogenous SOD1. A549 cells were transfected with either pFlag-SOD1 or pFlag vector. 48 hr after transfection, cells were infected with the wild-type (WT) or mutant (Mut△F2) virus for 24 h. (A) Viability of infected cells was determined by WST-1 assay. Absorbance (450 nm) indicates cell viability. Bar graphs represent mean ± SD from at least 6 independent experiments. Statistical significance was relative to the mock infection, and was determined using the *t*-test (*$p < 0.01$). (B) The Annexin V-FITC positive cells were analyzed by flow cytometry. Numbers in each highlighted quadrant reflect the percentage of the cells in apoptosis zone. Data are the means ± SD of at least three separate experiments. (C) A549 cells infected with the indicated virus and mock-infected cells were harvested for immunoblotting using antibodies against PARP, SOD1, and IAV-NP proteins. Pre-PARP and cleaved PARP (cPARP) were indicated by arrows.

(Fig. 3D). Therefore, these results strongly suggest that PB1-F2 is specifically implicated in the alteration of cellular redox state.

3.4. PB1-F2-mediated ROS is proportionally associated with host cell death

As described in Fig. 1, it was demonstrated that IAV-mediated ROS might play a role in the regulation of cell death. Further experiments were conducted to determine whether PB1-F2 could indeed stimulate ROS generation by down-regulating SOD1, and whether the enhanced levels of ROS could lead to cell death of the infected cells. To assess the function of PB1-F2 in cell death, A549 cells were infected with either wild-type or mutantΔF2 virus and then analyzed via WST-1 cell proliferation and Annexin-V apoptosis assay. At this time, exogenous SOD1 was transiently overexpressed to replenish the sequestered SOD1 protein. As shown in Fig. 4A, it appeared likely that virus infection itself largely lowered the cell viability, but overexpression of SOD1 or infection with the mutant virus reduced cell death at similar level. Fig. 4B shows that the mutantΔF2 virus had much weaker ability to induce apoptosis, whereas significant induction was observed for the wild-type virus. SOD1 expression in the cells infected with wild-type virus also displayed marked rescue from apoptosis. The attenuation of cell death due to exogenous expression of SOD1 may provide evidence that the enhanced levels of ROS due to the PB1-F2-mediated SOD1 decrease could be responsible for cell death in infected cells. In order to further support the critical role of PB1-F2 in ROS-associated apoptosis, the PARP cleavage activity between the two viruses and their relationship with SOD1 expression were compared. As demonstrated in Fig. 4C, expression of SOD1 or infection with mutantΔF2 virus both apparently led to diminished levels of cleaved PARP compared to the wild-type virus-infected cells. Furthermore, the level of IAV-NP expression was also proportional to the PARP cleavage activity. Thus, this experimental data may provide evidence that PB1-F2-mediated apoptosis is beneficial for the virus as a potential effector in early stages of the replication cycle.

4. Discussion

Apoptosis is a crucial function in both normal and dysregulated cells in a variety of biological processes. Although IAV infection is known to bring about severe cell death, it is likely that programmed cell death substantially contributes to viral propagation, as well as the pathogenesis associated with IAV infection [21]. IAV infection,

which is accompanied by apoptosis, is a major cause of severe respiratory diseases [4]. A number of studies have suggested a variety of mechanisms involving cell death during IAV infection [12,22]. Recent studies proposed an important role of oxidative stress in the infectivity and pathogenicity of IAV [1,23,24]. However, the mechanism underlying IAV-mediated ROS formation and its effect on apoptosis has been much less well understood.

According to recent studies on caspase activation, the onset of apoptosis due to IAV appears to be crucial for virus propagation [17]. It appears likely that IAV-mediated generation of ROS induces apoptotic cell death. Previously, we also demonstrated that IAV induced a specific Sp1-dependent decrease in SOD1 protein level, leading to an inverse relationship between SOD1 level and expression of viral polymerase PB1 at 24 hpi [10]. Our works demonstrated a unique mechanism by which IAV-mediated down-regulation of the major antioxidant SOD1 led to excess intracellular ROS. However, the mechanism by which the oxidative condition resulted in enhanced levels of the PB1 remains to be solved. The enhancement of ROS may establish conditions favorable for viral propagation as well as host apoptosis, thereby facilitating viral propagation subsequently followed by the lytic process. It is generally believed that viruses have their own genes to counteract the host antiviral state. That would be a likely mechanism to enhance viral yields independently of host gene activation. With these considerations, the present study thus attempted to determine whether the oxidative stress induced by the IAV-mediated SOD1 decrease is indeed responsible for induction of apoptosis, and whether these processes are attributable to a specific viral factor.

Among several viral gene segments, PB1-F2 was found to be involved in the IAV-induced down-regulation of SOD1. This wild-type PB1-F2-mediated SOD1 down-regulation was largely responsible for the increase in both superoxide anion and apoptosis, whereas disruption of PB1-F2 resulted in almost loss of the control ability. Inversely, the rescue of PB1-F2-induced cell death by exogenous SOD1 expression clearly evidenced the involvement of PB1-F2 in ROS generation and apoptosis. PB1-F2 contains a mitochondrial targeting sequence which interacts with the permeability transition pore complex to result in cytochrome C release [12,13]. Therefore, these results suggest that PB1-F2 is specifically involved in the alteration of the cellular redox state, and thereby support its role in the induction of apoptosis. However, this does not rule out the possible involvement of PB1-F2 in viral propagation via different mechanisms. Beside of its critical role in apoptosis, PB1-F2 has been shown to enhance the levels of viral proteins such as NP. In contrast, infection with mutantΔF2 virus revealed diminished activity in the induction of apoptosis, as well as decreased levels of viral NP expression. Therefore, this suggests that the mutantΔF2 virus may be an attenuated virus in A549 cells. Basically, IAV (A/WSN/1933) has the full length PB1-F2, which is an 87- to 90-amino-acid-long protein encoded by an alternate +1 ORF within the PB1 gene. However, other human influenza strains have invariably evolved to introduce truncations in the PB1-F2 ORF. The significance of this in pandemic strains remains unclear. Interestingly, viruses expressing different lengths of PB1-F2 revealed moderately different patterns of viral gene expression and histopathology early in infection [25], which is consistent with our findings. Furthermore, modulation of viral replication may occur through the involvement of PB1-F2 in activation of the viral polymerase immediately after virus infection. In related studies, we determined the functional difference of each variant PB1-F2 from isogenic virus strains and their correlation with virulence. Thus, the proportional regulation of ROS-associated apoptosis and viral gene expression by PB1-F2 transfection or wild-type IAV infection occurring within 24 hpi suggests that PB1-F2 might affect viral propagation through modulation of the redox state during early stages of the infection, which could ultimately contribute to the pathogenesis of IAV.

Conflict of interest

None.

Acknowledgment

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References

[1] E. Peterhans, M. Grob, T. Burge, R. Zanoni, Virus-induced formation of reactive oxygen intermediates in phagocytic cells, Free Radic. Res. Commun. 3 (1987) 39–46.
[2] F.M. Yakes, B.V. Houten, Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 514–519.
[3] F. Madeo, E. Fröhlich, M. Ligr, M. Grey, S.J. Sigrist, D.H. Wolf, K.-U. Fröhlich, Oxygen stress: a regulator of apoptosis in yeast, J. Cell. Biol. 17 (1999) 757–767.
[4] W. Chen, P.A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J.R. Bennink, J.W. Yewdell, A novel influenza A virus mitochondrial protein that induces cell death, Nat. Med. 7 (2001) 1306–1312.
[5] K.B. Schwarz, Oxidative stress during viral infection, Free Radic. Bio. Med. 21 (1996) 641–649.
[6] S.J. Kim, P.K.Y. Wong, ROS upregulation during the early phase of retroviral infection plays an important role in viral establishment in the host cell, J. Gen. Virol. 94 (2013) 2309–2317.
[7] M. Takahashi, M. Higuchi, G.N. Makokha, H. Matsuki, M. Yoshita, Y. Tanaka, M. Fujii, HTLV-1 tax oncoprotein stimulates ROS production and apoptosis in T cells by interacting with USP10, Blood 122 (2013) 715–725.
[8] T. Akaike, Y. Noguchi, S. Ijiri, K. Setoguchi, M. Suga, Y.M. Zheng, B. Dietzschold, H. Maeda, Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 2448–2453.
[9] P.F. Bove, A.v.d. Vliet, Nitric oxide and reactive nitrogen speciesin airway epithelial signaling and inflammation, Free Radic. Biol. Med. 41 (2006) 515–527.
[10] C.W. Pyo, N. Shin, K.I. Jung, J.H. Choi, S.Y. Choi, Alteration of copper-zinc superoxide dismutase 1 expression by influenza A virus is correlated with virus replication, Biochem. Biophys. Res. Commun. 450 (2014) 711–716.
[11] X. Wang, M. Li, H. Zheng, T. Muster, P. Palese, A. Beg, A. García-Sastre, Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon, J. Virol. 74 (2000) 11566–11573.
[12] J.S. Gibbs, D. Malide, F. Hornung, J.R. Bennink, J.W. Yewdell, The influenza A virus PB1-F2 protein targets the inner mitochondrial membrane via a predicted basic amphipathic helix that disrupts mitochondrial function, J. Virol. 77 (2003) 7214–7224.
[13] D. Zamarin, A. Garcia-Sastre, X. Xiao, R. Wang, P. Palese, Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1, PLoS Pathog. 1 (2005) e4.
[14] J.L. McAuley, K. Zhang, J.A. McCullers, The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis, J. Virol. 84 (2010) 558–564.
[15] R. Hai, M. Schmolke, Z.T. Varga, B. Manicassamy, T.T. Wang, J.A. Belser, M.B. Pearce, A. García-Sastre, T.M. Tumpey, P. Palese, PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models, J. Virol. 84 (2010) 4442–4450.
[16] N. Kumar, Y. Liang, T.G. Parslow, Y. Liang, Receptor tyrosine kinase inhibitors block multiple steps of influenza a virus replication, J. Virol. 85 (2011) 2818–2827.
[17] W.J. Wurzer, O. Planz, C. Ehrhardt, M. Giner, T. Silberzahn, S. Pleschka, S. Ludwig, Caspase 3 activation is essential for efficient influenza virus propagation, EMBO J. 22 (2003) 2717–2728.
[18] K. Chen, P. Iribarren, J. Hu, J. Chen, W. Gong, E.H. Cho, S. Lockett, N.M. Dunlop, J.M. Wang, Activation of toll-like receptor 2 on microglia promotes cell uptake of Alzheimer disease-associated amyloid β peptide, J. Biol. Chem. 281 (2006) 3651–3659.
[19] C.W. Pyo, J.H. Choi, S.M. Oh, S.Y. Choi, Oxidative stress-induced cyclin D1 depletion and its role in cell cycle processing, Biochim. Biophys. Acta 1830 (2013) 5316–5325.
[20] N.K. Yoo, C.W. Pyo, Y. Kim, B.Y. Ahn, S.Y. Choi, Vaccinia virus-mediated cell cycle alteration involves inactivation of tumour suppressors associated with Brf1 and TBP, Cell. Microbiol. 10 (2008) 583–592.

[21] S. Herold, M. Steinmueller, W. von Wulffen, L. Cakarova, R. Pinto, S. Pleschka, M. Mack, W.A. Kuziel, N. Corazza, T. Brunner, W. Seeger, J. Lohmeyer, Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF- related apoptosis-inducing ligand, J. Exp. Med. 205 (2008) 3065–3077.

[22] E.C. Roberson, J.E. Tully, A.S. Guala, J.N. Reiss, K.E. Godburn, D.A. Pociask, J.F. Alcorn, D.W. Riches, O. Dienz, Y.M. Janssen-Heininger, V. Anathy, Influenza induces endoplasmic reticulum stress, caspase-12-dependent apoptosis, and c-Jun N-terminal kinase-mediated transforming growth factor-beta release in lung epithelial cells, Am. J. Respir. Cell. Mol. Biol. 46 (2012) 573–581.

[23] T. Hennet, E. Peterhans, R. Stocker, Alterations in antioxidant defences in lung and liver of mice infected with influenza A virus, J. Gen. Virol. 73 (1992) 39–46.

[24] S. Christen, E. Peterhans, R. Stocker, Antioxidant activities of some tryptophan metabolites: possible implication for inflammatory diseases, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 2506–2510.

[25] L. Pena, A.L. Vincent, C.L. Loving, J.N. Henningson, K.M. Lager, A. Lorusso, D.R. Perez, Restored PB1-F2 in the 2009 pandemic H1N1 influenza virus has minimal effects in swine, J. Virol. 86 (2012) 5523–5532.