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An inhibitory effect of A20 on NF-κB activation in airway epithelium upon influenza virus infection

Akira Onose [a], Shu Hashimoto [a,*], Shinichi Hayashi [a], Shuichiro Maruoka [a], Fumio Kumasawa [a], Kenji Mizumura [a], Itsuro Jibiki [a], Ken Matsumoto [a], Yasuhiro Gon [a], Tomoko Kobayashi [a], Noriaki Takahashi [a], Yasuko Shibata [b], Yoshimitsu Abiko [b], Toshikatsu Shibata [c], Kazufumi Shimizu [c], Takashi Horie [a]

[a] *Division of Respiratory Medicine, Department of Internal Medicine, Nihon University School of Medicine, 30-1 Oyaguchikamimachi, Itabashi-ku, Tokyo 173-8610, Japan*
[b] *Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo, Japan*
[c] *Department of Microbiology, Nihon University School of Medicine, Japan*

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Abstract

Influenza is a major disease in humans. The reemergence of avian influenza A viruses has indicated that hyperinflammatory responses are closely related to the severity of disease. Influenza virus infection induces nuclear transcription factor kappaB (NF-κB) activation. NF-κB and NF-κB-dependent gene products promote lung inflammation and injury. Therefore, it is important to investigate the means to attenuate NF-κB activation. A20 is a cytoplasmic zinc finger protein that inhibits NF-κB activity, However, little is known about the role of A20 in influenza virus infection. Here, we have examined the role of A20 in influenza virus infection-induced NF-κB promoter activation in human bronchial epithelial cells. The results showed that (1) A20 protein and mRNA are inducible and expressed in the lung from mice and human bronchial epithelial cells upon influenza virus infection; (2) NF-κB promoter activation was induced in bronchial epithelial cells upon influenza virus infection; and (3) overexpression by transient transfection of A20 attenuated NF-κB promoter activation in bronchial epithelial cells. These results indicate that A20 may function as a negative regulator of NF-κB-mediated lung inflammation and injury upon influenza virus infection, thereby protecting the host against inflammatory response to influenza virus infection.

Keywords: A20; Influenza virus; NF-κB; Bronchial epithelium; Inflammation

1. Introduction

Influenza virus infection is an acute respiratory infection and one of the most pandemic infectious diseases (Thompson et al., 2003). Highly pathogenic avian influenza virus subtypes H5N1 and H7N7 caused acute respiratory illness including fetal disease (Shortridge, 2003). New strains of influenza A viruses emerge in humans. Vaccine and anti-neuraminidase inhibitors are widely used for the prevention and treatment of influenza virus infection, but it is not logically possible to prepare vaccines against all strains of influenza virus, especially unexpected outbreak of non-human subtypes of influenza A viruses (Webby and Webster, 2003; Cheng et al., 2004; Gubareva et al., 2000). The most severe complications are viral pneumonia and lung injury (Yuen et al., 1998; Peiris et al., 2004; Tran et al., 2004; Fouchier et al., 2004). During the development of viral pneumonia and lung injury, influenza virus infection-associated proinflammatory responses are involved in the pathogenesis of viral pneumonia and lung injury (Peiris et al., 2004; Guan et al., 2004; Cheung et al., 2002; Van Reeth, 2000). Therefore, it is important to clarify how to attenuate and terminate proinflammatory responses.

* Corresponding author. Tel.: +81 3 3972 8111; fax: +81 3 3972 2893.
E-mail address: shuh@med.nihon-u.ac.jp (S. Hashimoto).

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Activation of the nuclear transcription factor kappa-B (NF-κB) is a hallmark of inflammatory processes induced by various stimuli, including viral pathogens (Fan et al., 2001; Santoro et al., 2003). NF-κB activation during viral infection has been interpreted as a protective response of the host against viral infection (Santoro et al., 2003). NF-κB regulates anti-viral cytokine, interferon (IFN) and tumor necrosis factor (TNF)-α against influenza virus infection (Santoro et al., 2003; Seo and Webster, 2002; Julkunen et al., 2000). In contrast, recent data have indicated that NF-κB activity is associated with influenza virus infectivity and propagation (Nimmerjahn et al., 2004; Wurzer et al., 2004). Therefore, it is important to investigate the regulatory mechanism in NF-κB activity induced upon influenza virus infection. Preliminary data on comprehensive analysis of gene expression in human bronchial epithelial cells upon influenza virus infection have shown that A20 mRNA expression is up-regulated in bronchial epithelial cells upon influenza virus infection.

The cytoplasmic zinc finger protein A20 is encoded by an immediate early response gene and originally identified as a TNF-α and interleukin (IL)-1-inducible gene product in endothelial cells (Song et al., 1996). A20 acts as an inhibitor of NF-κB activation and NF-κB-dependent gene expression (Gon et al., 2004; Hu et al., 1998). The expression of A20 is itself under the control of NF-κB (Krikos et al., 1992), suggesting that A20 is involved in a negative feedback regulation of NF-κB activation and NF-κB-dependent gene expression. Therefore, it is possible that A20 may be a negative regulator of influenza virus infection-induced inflammatory responses. However, the role of A20 in inflammatory responses has not been clarified. In order to do so, we examined the role of A20 in NF-κB activation in human airway epithelial cells upon influenza virus infectionin this study.

2. Materials and methods

2.1. Virus, mice and cells

Influenza virus A/Udon/307/72 (H3N2) and A/PR/8/34 (H1N1) were grown as previously described (Kujime et al., 2000). Five- to six-week-old C57/BL mice were purchased from Oriental Co., Inc. (Tokyo, Japan). Mice were housed in pathogen free conditions in accordance with the animal care guidelines of Nihon University School of Medicine. Bronchial epithelial cell lines, NCI-H_{292}, and human embryonic kidney cell lines, HEK293, were obtained from American Type Culture as previously described (Gon et al., 2004).

2.2. Affymetrix gene chip analysis

Serum-starved bronchial epithelial cells were infected with influenza virus (A/Udon/307/72) at multiplicity of infection (moi) of 5 and cultured for 6 h. Total RNA was isolated from the harvested cells using RNeasy Mini Kit (Qiagen, Valencia, CA). Preparations of biotin-labeled cRNAs and hybridization to the HU-U95A Gene Chip set (Affymetrix, Santa Clara, CA) were performed according to the manufacturer's instructions. Prior to the hybridization to the HU-U95A Gene Chip, cRNAs were first hybridized to the Test 3 chip to confirm that differences in housekeeping genes between the samples were less than 10%.

2.3. A20 mRNA and protein expression

For analysis of A20 mRNA expression in bronchial epithelial cells, serum-starved bronchial epithelial cells were infected with influenza virus (A/Udon/307/72) at moi of 5 and cultured. A20 mRNA and G6PDH mRNA expression were determined at the time indicated after influenza virus infection. For analysis of A20 mRNA and protein expression in the lung from mice, the mice were intranasally inoculated with 50 μl of 10^4 PFU of influenza virus (A/PR/8/34) or 50 μl of PBS (control mice). The mice were anesthetized and sacrificed, and the lungs were removed at the desired times as indicated after infection. For quantitative PCR analysis of mRNA expression, total mRNA was isolated from BEC and homogenized lung from mice using an RNA gents total RNA isolation system (Promega, Madison, WI) and reverse transcribed with Improm-II reverse transcriptase (Promega). Primers for each A20 were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). The following oligonucleotides were used: human A20 sense, 5′-CTGCCCAGGAATGCTACAGATAC-3′; human A20 anti-sense, 5′-GTGGAACAGCTCGGATTTCAG-3′; mouse A20 sense, 5′-AAGCTTCTAAAGGAGTACTTGATAGTGATG-3′; mouse A20 anti-sense, 5′-CAATTTTGCAGCGTTGATCAG-3′. Quantitative RT-PCR was carried out by an ABI PRISM 7300 sequence detection system using SYBR Green PCR Master Mix (Applied Biosystems). Immunohistochemical analysis of A20 protein expression was performed using immunoperoxidase technique as previously described (Koura et al., 2000). The primary antibody was rabbit anti-human A20 polyclonal antibody (Santa Cruz, Santa Cruz, CA) and nonimmune normal rabbit IgG was used as a negative control. Counterstaining was performed with haematoxylin.

2.4. Western blot analysis of IκB phoshorylation and total IκB

Serum-starved bronchial epithelial cells were infected with influenza virus (A/Udon/307/72) at moi of 5 and were then cultured for desired times as indicated after infection with influenza virus. Western blotting analysis of IκBα phosphorylation and degradation was performed using rabbit polyclonal antibody to phosphorylated IκB (Promega) and rabbit polyclonal antibody to IκB (Promega). β̃ Tubulin was used as a housekeeping protein.

2.5. Plasmids, transfection and luciferase reporter assay

For analysis of NF-κB luciferase activity, serum-starved bronchial epithelial cells, which had been transiently transfected by NF-κB-Luc reporter plasmid, were infected with influenza virus (A/Udon/307/72) at moi of 5 and were then cultured for the desired time as indicated. For analysis of the effect of A20,

serum-starved bronchial epithelial cells were transiently transfected with the pcDNA3-A20 expression vectors (Miller et al., 1997) using FuGENE6 (Roche Diagnostics Corp., Indianapolis, IN). The total amount of cDNA was kept constant by supplementation with empty vector, pcDNA3 (Invitrogen, Carlsbad, CA). Every transfection included 500 ng of NF-κB-Luc reporter plasmid (Stratagene, La Jolla, CA) together with 5 ng of pRL-SV40-Renilla for normalization of transfection efficiency. After 24 h with the transfection, bronchial epithelial cells were infected with influenza virus (A/Udon/307/72) at moi of 5 and NF-κB reporter activity was determined at the desired times as indicated after infection with influenza virus as previously described (Jibiki et al., 2003).

2.6. Virus protein synthesis

Influenza virus protein synthesis was determined by Western blotting using the polyclonal anti-influenza virus serum as previously described (Maruoka et al., 2003). Briefly, HEK293 cells were transiently transfected with pcDNA3-Myc-A20 (2 μg) or pcDNA3 (2 μg) as control. At 24 h after transfection, the cells were infected with influenza virus (A/Udon/307/72) at moi of 5 for the desired times as indicated. At the end of cultivation, cells were then lysed and immunoblotted with antibodies to polyclonal anti-influenza virus (1:1000 dilution for overnight at 4) and anti-myc antibody (9E10) as previously described (Maruoka et al., 2003). Anti-rabbit–horseradish peroxidase antibody was used as a second antibody (1:2000 dilution for 1 h at room temperature). The antibody-antigen complexes were detected using the ECL system (Amersham-Pharmacia Biotech). The appropriate expressions of Myc-A20 were confirmed in the lysate.

2.7. Statistical analysis

Statistical significance was analyzed by using analysis of variance (ANOVA). *P* value of less than 0.05 was considered significant.

3. Results

3.1. Comprehensive gene expression in bronchial epithelial cells upon influenza virus infection

For each gene on the chip-reading software makes an expression call, such as “present”, “absent” or “marginal” according to the difference between fluorescence intensity in perfectly matched sequences and mismatched sequences containing a single base pair mutation. To minimize the number of false positive results, we excluded the sequences whose expression call of sample infected with influenza virus was determined as “absent”. We were able to detect 5998 genes among the 12,000 genes represented in the chip. We made three criteria for the list that show genes induced by influenza virus infection: (1) the intensity of expression was with at least a 10-fold increase by influenza virus infection, (2) mean expression intensity, determined by the average difference between fluorescence in perfectly matched sequences and mismatched sequences, in the sample infected with influenza virus, was greater than 100 (because low intense results were sometimes inaccurate), and (3) expression difference between untreated cells and influenza virus infected cells was statistically significant. Table 1 represents the mRNA expression level indicated as the average difference in only medium treated or influenza virus infected cells. When using these criteria, influenza virus infection was found to up-regulate the expressions of 32 genes. Among these genes, A20 is a remarkable gene that inhibits NF-κB activity. Therefore, the study was conducted to examine the effect of A20 in NF-κB activity induced upon influenza virus infection.

Table 1

GeneBank accession no.	Gene description	Uninfected intensity	IV infected intensity	Fold increase
AF008445	Phospholipid scramblase	446	10,992	24.6
M30818	Interferon-induced cellular resistance mediator protein (MxB)	735	16,876	23.0
X04602	Interleukin BSF-2 (B-cell differentiation factor)	61	1392	22.8
M33882	p78 protein mRNA (MxA)	874	19,923	22.8
U52513	RIG-G mRNA	873	19,595	22.4
X02875	Oligo A synthetase E	892	18,356	20.6
U72882	Interferon-induced leucine zipper protein (IFP35)	986	18,048	18.3
M13755	Interferon-induced 17-kDa/15-kDa protein	909	14,383	15.8
M55153	Transglutaminase (TGase)	759	10,685	14.1
M24594	Interferon-inducible 56-kDa protein	1090	15,211	14.0
U59877	Low-Mr GTP-binding protein (RAB31) mRNA	629	7332	11.7
M87434	71-kDa 2 5 oligoadenylate synthetase (p69 2-5A synthetase)	1063	11,878	11.2
D90070	ATL-derived PMA-responsive (APR) peptide	195	2166	11.1
S82240	RhoE=26-kDa GTPase homolog	157	1738	11.1
U18671	Stat2 gene	424	4641	10.9
U77643	K12 protein precursor	1475	15,632	10.6
U15932	Dual-specificity protein phosphatase mRNA	1065	11,256	10.6
M59465	Tumor necrosis factor-α inducible protein A20 mRNA	353	3717	10.5
U51010	Nicotinamine *N*-methyltansferase	951	9826	10.3
X13839	Vascular smooth muscle α-actin	81	829	10.2

3.2. A20 mRNA expression in human bronchial epithelial cells and lung

To verify the data on A20 mRNA expression obtained by Gene Chip analysis, we quantitatively examined A20 mRNA expression in bronchial epithelial cells and the lung from mice upon influenza virus infection using quantitative RT-PCR.

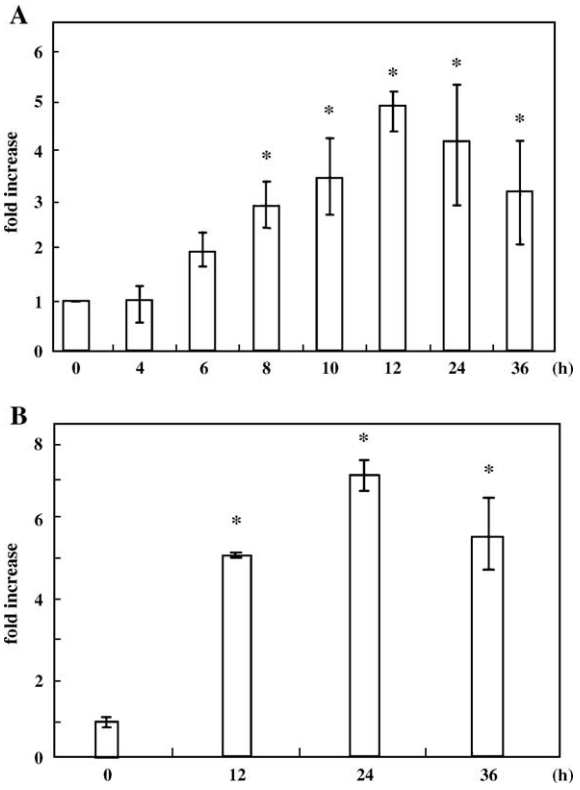

Fig. 1. A20 mRNA expression in human bronchial epithelial cells and in lung homogenates from mice. A20 mRNA expression in bronchial epithelial cells (A) and in the lungs from mice (B) were analyzed at times as indicated after influenza virus infection by quantitave RT-PCR amplification. The results are expressed as means±S.D. of three different experiments. * indicates $P<0.05$ compared with mRNA levels in influenza virus-uninfected bronchial epithelial cells (A). * indicates $P<0.05$ compared with mRNA levels in the lung from influenza virus-uninfected mice (B).

A20 mRNA expression in bronchial epithelial cells increased at 8 h, sustained from 8 to 12 h, and thereafter decreased at 24 h (Fig. 1A). A20 mRNA expression in the homogenized lung from mice increased at 12 h and was maximal at 24 h and thereafter decreased at 36 h (Fig. 1B). These results indicate that A20 mRNA is inducible and expressed in the lung from mice and bronchial epithelial cells in a time-dependent manner.

3.3. A20 protein expression in the lung

To verify the data on A20 mRNA expression at the levels of protein expression and identify the cellular source of A20, we performed immunohistochemistry on lung tissue from mice upon influenza virus A/PR/8 infection. We stained lung tissue specimens with anti-A20 antibody. Representative results are shown in Fig. 2. A20 is positively stained in the bronchial epithelial cells and alveolar epithelial cells of lung specimens from influenza virus-infected mice by anti-A20 antibody (Fig. 2A), but not in those from influenza virus-uninfected mice (Fig. 2C). The lung tissue specimens of lung from either influenza virus infected or uninfected mice were not stained with control antibody, normal rabbit IgG (Fig. 2B and D).

3.4. Influenza virus infection induces IκB phosphorylation and NF-κB activation in human bronchial epithelial cells

In the next series of experiments, we examined the effect of A20 on NF-κB activity. To this end, we first examined NF-κB signaling in bronchial epithelial cells upon influenza virus infection. Among the many proteins exhibiting IκB function, IκBα is the only inhibitor that, in response to cell stimulation, dissociates from the NF-κB heterodimer complex, with kinetics matching that of the translocation of NF-κB to the nucleus. It has therefore been suggested that the inducible activation of NF-κB is regulated mainly by the dissociation of NF-κB an IκBα (Traenckner et al., 1995). In order to examine influenza virus infection-induced NF-κB signaling, we examined IκB phosphorylation and IκB degradation, and NF-κB promoter activation in bronchial epithelial cells upon influenza virus infection. As shown in Fig. 3A, amounts of phosphorylated IκB in influenza virus-infected bronchial epithelial cells increased at 4 h after influenza virus infection, were maximal at 8 h and thereafter slightly decreased at 10 h (Fig. 3A, upper panel, P-IκBα). Amounts of total IκB protein decreased along with time of culture periods, indicating an increase in amounts of phosphorylated IκB proteins inversely correlated with a decrease in amounts of total IκB proteins (Fig. 3A, middle panel, IκBα). Almost equal amounts of tubulin protein were blotted with anti-tubulin antibody regardless of the time of culture periods (Fig. 3A, lower panel, β-tubulin). Next, the cells that had been transiently transfected by NF-κB-Luc reporter plasmid were infected with influenza virus and then NF-κB-dependent luciferase gene activity was determined at various times as indicated after infection with influenza virus. NF-κB reporter activity in influenza virus-infected cells increased at 4 h, was maximal at 8 h and thereafter returned to the basal levels at 24 h (Fig. 3B).

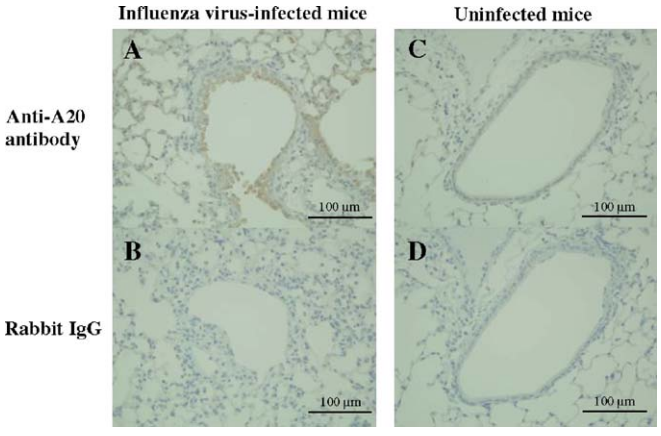

Fig. 2. A20 expression in the lung tissue specimens from mice. Mice were intranasallly inoculated with influenza virus or PBS (control mice) and the lungs were removed on day 4. The lung specimens from influenza virus-infected mice (A and B) and control mice (C and D) were stained with anti-A20 antibody (A and C) or control rabbit IgG (B and D). Original magnification: ×400. The results are representative of each group.

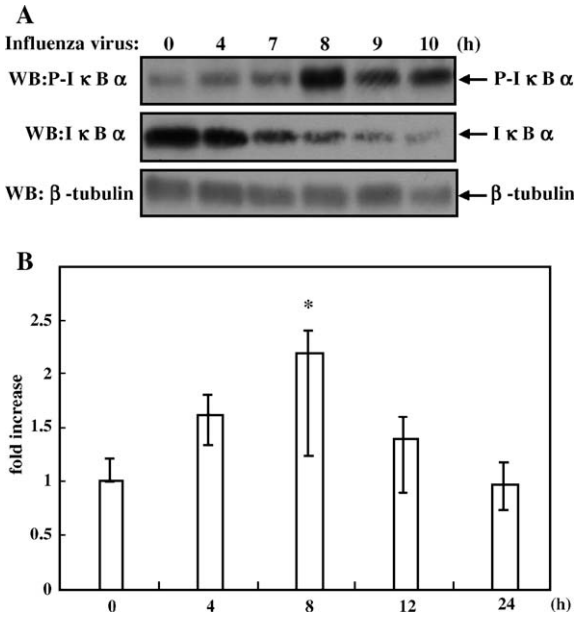

Fig. 3. Influenza virus infection induces IκB phosphorylation and NF-κB activation in human bronchial epithelial cells. IκBα phosphorylation (P-IκBα), IκBα degradation (IκBα) and β-tubulin protein expression were analyzed at times as indicated after influenza virus infection (A). NF-κB-dependent luciferase gene activity was determined at times as indicated after influenza virus infection (B). The results are expressed as means ± S.D. of three different experiments. * indicates $P<0.05$ compared with NF-κB activity in influenza virus-uninfected cells.

3.5. Transfection of A20 attenuates influenza virus infection-induced NF-κB promoter activation

Finally, we examined the effect of A20 in influenza virus infection-induced NF-κB promoter activation. The bronchial epithelial cells were transiently transfected by the A20 expression vector. Transient transfection of A20 attenuated

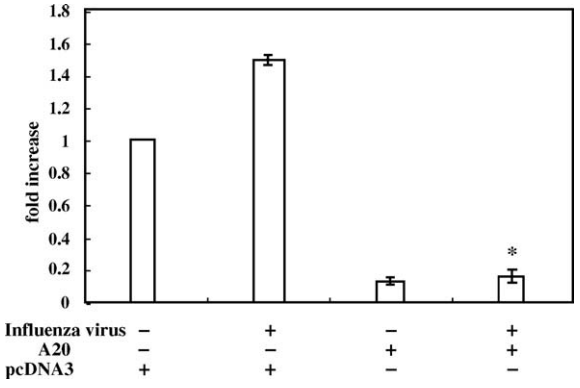

Fig. 4. NF-κB activity is depressed in the A20-transfected human bronchial epithelial cells. Bronchial epithelial cells were transiently co-transfected with the human NF-κB-Luc reporter plasmid as well as either the pcDNA-A20 expression vector or the empty pcDNA3 vector. After 24 h with the transfection, bronchial epithelial cells were infected with influenza virus and NF-κB reporter activity was determined at the desired times as indicated after influenza virus infection. The results are expressed as means ± S.D. of three different experiments. * indicates $P<0.05$ compared with NF-κB promoter activity in empty pcDNA3 vector-transfected cells.

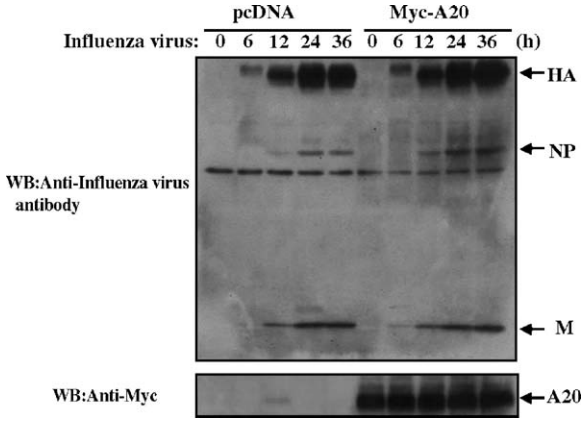

Fig. 5. Influenza virus protein synthesis in A20-transfected HEK293. Influenza virus protein synthesis was analyzed by Western blotting using polyclonal anti-influenza virus antibody as described in Materials and methods. The results are representative of two independent experiments.

NF-κB promoter activation in bronchial epithelial cells infected with influenza virus (Fig. 4).

3.6. Influenza virus protein synthesis in A20-transfected cells

Transient transfection of A20 into bronchial epithelial cells resulted in the inhibition of NF-κB promoter activation upon influenza virus infection. If influenza virus growth is inhibited in A20-transfected bronchial epithelial cells compared to that in control vector-transfected bronchial epithelial cells, the inhibited NF-κB activation might result from the inhibited replication of influenza virus. To test this possibility, influenza virus protein expression in A20-transfected HEK293 versus control vector-transfected HEK293 was compared. The amounts of tree major influenza virus protein expression, including HA, NP and M1 in A20-transfected HEK293, were comparable to those in control vector-transfected HEK293, indicating that influenza virus replication is not inhibited in A20-transfected (Fig. 5). These results indicated that A20 doses not inhibit influenza virus replication in these experimental conditions. The total number of cells and cell viability determined by trypan blue exclusion dye at the end of the culture period of each experiment (indicated in Figs. 1–5) did not differ with culture conditions (data not shown).

4. Discussion

In this study, in order to clarify the potentially protective role of A20 in lung inflammation and injury upon influenza virus infection, we examined the inducible expression of A20 protein and mRNA in the lung from mice and in bronchial epithelial cells and the role of A20 in NF-κB promoter activation in bronchial epithelial cells. The results showed that A20 expression was induced in the lung from mice and bronchial epithelial cells, and overexpression of A20 attenuated NF-κB promoter activation in bronchial epithelial cells upon influenza virus infection. These results indicate that A20 may function as a negative regulator for inflammatory response in influenza virus infection.

There are two major means to control influenza virus infection: the inhibition of virus propagation and virus infection-associated harmful events, including pulmonary inflammation and injury. A20 is a cytoplasmic zinc finger protein originally identified as a TNFα- and IL-1-inducible gene product in endothelial cells (Song et al., 1996). Subsequently, A20 expression is induced in a variety of cell types upon various stimuli (Krikos et al., 1992; Becker et al., 2000; Opipari et al., 1990; Jaattela et al., 1996). A20 negatively regulates NF-κB signaling (Gon et al., 2004). However, the role of A20 in influenza virus infection-induced NF-κB activity in bronchial epithelial cells has not been determined. Here, we have shown that A20 is inducible and expressed in the lung from mice and in bronchial epithelial cells upon influenza virus infection. To clarify the role of A20 in NF-κB activity, we transiently transfected A20 into bronchial epithelial cells and examined the role of overexpressed A20 in NF-κB promoter activation in bronchial epithelial cells upon influenza virus infection. The results have shown that overexpression of A20 results in the attenuation of influenza virus infection-induced NF-κB promoter activation.

We must carefully interpret our results when extrapolating in vitro data of the inhibitory effect of A20 on NF-κB activation to the in vivo. Airway epithelial cells produce IFN-β and TNF-α that exert anti-viral activity at the early stage of influenza virus infection (Santoro et al., 2003; Seo and Webster, 2002; Julkunen et al., 2000). They serve as the key player in the host defense against influenza virus infection. The promoter of IFN-β gene and TNF-α gene contains an NF-κB-binding site and NF-κB regulates IFN-β and TNF-α expression (Santoro et al., 2003; Seo and Webster, 2002; Julkunen et al., 2000). Accumulated cells including macrophages, T and B lymphocytes, natural killer cells and neutrophils at the site of viral infection are capable of producing IFN-αγ, TNF-α, IL-1 and IL-18 (Kujime et al., 2000; Tamura and Kurata, 2004). These cytokines promote the development of Th1-type responses that are a prerequisite for antiviral immunity. NF-κB regulates the gene expression of these cytokines. Thus, NF-κB plays a defensive role against influenza virus infection.

In the contrast, NF-κB is suggested to be harmful to the host defense against influenza virus infection (Nimmerjahn et al., 2004; Wurzer et al., 2004; Ichiyama et al., 2004). NF-κB activity enhances virus propagation (Wurzer et al., 2004). Alveolar type II epithelial cells with low NF-κB activity were resistant to influenza A virus infection, whereas the cells with high NF-κB activity became susceptible to infection (Nimmerjahn et al., 2004). These studies indicate that the infectivity of influenza virus is dependent on active NF-κB pathways. Influenza virus-associated encephalopathy is suggested to be a proinflammatory disease and affected patients exhibit high morbidity and mortality. The levels of proinflammatory cytokines, such as IL-6 in serum and NF-κB activity in peripheral blood mononuclear cells, are closely associated with disease severity (Ichiyama et al., 2004). Avian influenza A virus infection causes unusual severity of disease in humans. It has been indicated that hyperproduction of NF-κB-dependent cytokines including TNF-α and IP-10 may contribute to the unusual severity of human influenza H5N1 disease (Yuen et al., 1998; Peiris et al., 2004; Tran et al., 2004; Fouchier et al., 2004). In the murine model of viral pneumonia and lung injury upon influenza virus infection, MIP-2 that is a murine counterpart of human IL-8 increased in the lung tissue on day 2 after infection with influenza virus accompanied with increased number of neutrophils in the lung (Ochiai et al., 1993). Neutralization of MIP-2 activity with anti-MIP-2 antibody or immunoglobulin prevents and attenuates lung injury and myocarditis upon influenza virus infection (Kishimoto et al., 2004). Therefore, an inhibition of IL-8 production is an important strategy to attenuate and minimize pulmonary inflammation upon influenza virus infection. Taken together, NF-κB activity might be harmful and should be down-regulated when it is inappropriately and excessively activated. Thus, NF-κB plays the host defense and also promotes proinflammatory responses against influenza virus infection.

Vaccine is the key to controlling a pandemic and antiviral drugs are effective in treating human influenza virus disease (Palese, 2004). Recently, short interfering RNAs (siRNA) specific for highly conserved region influenza genes have been shown to inhibit influenza virus A replication in the lung from mice infected with influenza A virus (Tompkins et al., 2004). In addition to developing new antiviral drugs and vaccines, the means to control hyperinflammatory responses in the host has been expected. The inhibition of NF-κB by A20 may or may not be advantageous to the host defense mechanism against influenza virus infection. Recent data have indicated that A20 is critical to limiting inflammatory responses and host damages in multiple tissues (Lee et al., 2000; Heyninck and Beyaert, 2005). In this study, we showed the negative regulatory role of A20 in NF-κB activity in airway epithelial cell upon influenza virus infection. A20 may be advantageous to the host defense against influenza virus infection. However, the precise role of A20 remains to be clarified.

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