Arch Virol (2004) 149: 17–34
DOI 10.1007/s00705-003-0208-4

Archives of Virology
Printed in Austria

Induction of interferon-inducible protein-10 and monokine induced by interferon-γ from human endothelial cells infected with *Influenza A virus*

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Received November 8, 2002; accepted August 1, 2003
Published online October 20, 2003

Summary. Primary human umbilical vein endothelial cells (HUVECs) were infected with Influenza virus A/Aichi/2/68 (H3N2) in order to determine the role of endothelial cells in mediating inflammation induced upon virus infection. Structural proteins of the virus and mRNA of the M2 protein were detected in the infected cells, indicating that virus infection had occurred in HUVECs. The *Influenza A virus*-infected HUVECs showed elevated levels of gene expression of interferon (IFN)-inducible protein (IP)-10 and monokine induced by IFN-γ (Mig), while heat-, formalin- and diethyl ether-inactivated viruses did not enhance the IP-10 and Mig gene expression. The results thus indicate that infection of live *Influenza A virus* is responsible for elevation of IP-10 and Mig gene expression. The elevation of IP-10 and Mig gene expression in infected HUVECs was not accompanied by the elevation of IFN-γ gene expression, indicating that the elevation of IP-10 and Mig gene expression was independent of the IFN-γ pathway.

Introduction

Influenza A viruses cause respiratory tract infection and develop various complications in humans, especially during pandemics [53]. Involvement of a wide spectrum of the central nervous system (CNS) has also been observed during *Influenza A virus* infection [15]. Recently, quite a few cases of rapid progressive encephalitis/encephalopathy have been reported [15, 47, 58]. Edematous changes in the brain and intravascular thrombus formation suggest damage of endothelial

cells, destruction of the blood-brain barrier and subsequent vascular leakage in encephalitis/encephalopathy patients [16, 54, 56]. However little is known about the role of human endothelial cells in the pathogenesis of encephalitis/encephalopathy.

Many studies have been carried out to determine the functional contribution of chemokines to inflammation [32]. Interferon (IFN)-inducible protein (IP)-10 is a non-ELR (glutamic acid-leucine-arginine) CXC chemokine that has been shown to be a potent chemoattractant for activated T cells and NK cells by binding to the receptor CXCR3 [14, 40]. IP-10 has been shown to be expressed at an early stage within the CNS in response to mouse adenovirus type-1, *Lymphocytic choriomeningitis virus*, *Borna disease virus*, Theiler's murine encephalomyelitis virus and mouse hepatitis virus infections [3, 8, 20, 25, 51], suggesting that IP-10 plays an important role in host defense by serving to initiate and maintain an inflammatory response [4]. These observations led us to investigate the chemokine expression of endothelial cells upon infection with *Influenza A virus*.

Influenza A viruses replicate in epithelial cells and leukocytes, resulting in the production of chemokines and cytokines. Epithelial cells produce interleukin (IL)-6, IL-8 and RANTES (regulated upon activation, normal T-cell expressed and presumably secreted) in response to *Influenza A virus* infection [1, 35]. It has been reported that *Influenza A virus*-infected monocytes/macrophages secrete IFN-α/β, IL-1β, IL-6, IL-18, tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-3α RANTES, monocyte chemotactic protein (MCP)-1, MCP-3 and IP-10 [6, 7, 18, 34, 36, 41, 45, 50, 52]. Although it is known that human endothelial cells also produce IL-6 in response to *Influenza A virus* infection [59], it is not clear whether chemokines are expressed in endothelial cells infected with *Influenza A virus*. In the present study, we assessed the susceptibility of human endothelial cells to *Influenza A virus*. We then examined the profiles of cytokine and chemokine gene expressions in human umbilical vein endothelial cells (HUVECs) infected with *Influenza A virus*. We found that the IP-10 and monokine induced by IFN-γ (Mig) gene expression was enhanced as well as that of IL-6.

Materials and methods

Cells

HUVECs were purchased from Kurabo Industries, Ltd. (Osaka, Japan) and maintained in the medium supplied by the manufacturer, which contained 2% fetal calf serum, 10 ng/ml human endothelial growth factor, 1 μg/ml hydrocortisone, 5 ng/ml human fibroblast growth factor basic, 10 μg/ml heparin and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (MEM) with 10% calf serum and antibiotics.

Virus preparation

Influenza virus A/Aichi/2/68 (H3N2) and influenza virus A/PR/8/34 (H1N1) were used. The supernatant of influenza virus-infected MDCK cells cultured in MEM with 1 μg/ml trypsin was harvested at 48 h after inoculation and used for infection. The propagated influenza virus A/Aichi/2/68 (H3N2) in MEM was inactivated with heat at 65 °C for 60 min. Uninfected MDCK cells were cultured in MEM with trypsin and the supernatant was also collected

and used as sample of mock infection. A seed stock of influenza virus A/Aichi/2/68 (H3N2) was grown in the allantoic cavities of 11-day-old embryonated chicken eggs and purified by differential centrifugation and sedimentation through a sucrose gradient [23]. The purified virus was inactivated with heat at 65 °C for 60 min, 0.1% formalin, or 50% diethyl ether [27]. Purified live and inactivated viruses were used to certify the infectivity to HUVECs and the chemokine production. The virus was titrated on MDCK cells by plaque assay as described previously [5].

Chemicals

Cycloheximide (CHX) was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Infection of HUVECs with influenza virus

The virus was inoculated onto semi-confluent HUVECs in a T25 tissue culture flask at a multiplicity of infection (MOI) of 1 at room temperature. The inoculum was removed after 1 h. After washing with phosphate-buffered saline (PBS), a fresh medium was added to the flask, and the cells were incubated in a 5% CO_2 humidified incubator at 37 °C.

Antibodies

Chicken polyclonal antiserum to influenza A/Aichi/2/68 (H3N2) and mouse monoclonal antibody (L164/1) specific to hemagglutinin (HA) were used as a primary antibody in Western blot analysis [23]. Horseradish peroxidase-conjugated goat anti-chicken IgG polyclonal and goat anti-mouse IgG polyclonal antibodies were purchased from Bethyl Laboratories, Inc. (Montgomery, TX).

Western blot analysis

Cells were lysed with sodium dodecyl sulfate (SDS), and the lysate of 1×10^5 cells equivalents was subjected to an SDS-12% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (for chicken polyclonal antibody) or non-reducing conditions (for mouse monoclonal antibody) [49]. The separated proteins were electrotransferred onto a nitrocellulose membrane. The antibodies were allowed to bind to the filter and then to react with horseradish peroxidase-conjugated goat anti-chicken IgG or goat anti-mouse IgG polyclonal antibodies, and the proteins were detected by a chemiluminescent method (ECL Western Blotting Detection Reagents, Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated [9]. cDNA was synthesized from 2 μg of total RNA in a 14-μl reaction solution using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instruction. After incubation for 1 h at 42 °C, the samples were diluted to 45 μl with low TE buffer (10 mM Tris-HCl [pH 7.5] and 0.1 mM EDTA). One μl was used for the 30-μl PCR solution containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM dNTP, 0.4 μM primers and 1 U Ampli*Taq* DNA polymerase (Applied Biosystems, Foster City, CA). Primer design and PCR conditions are shown in Table 1. Eight μl from each reaction solution was electrophoresed on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

Table 1. PCR primer pairs and PCR conditions used in this study

Gene	GenBank accession no.		Primer pair	Sequence number	Size of product	PCR condition
Cytokines						
IL-1β	NM_000576	sense	5′-AGCACCTTCTTTCCCTTCATC-3′	357–377	447 bp	(1)
		antisense	5′-GCTGATGTACCAGTTGGGGA-3′	784–803		
IL-2	S77834	sense	5′-ATGTACAGGATGCAACTCCTGTCTT-3′	48–72	458 bp	(1)
		antisense	5′-GTTAGTGTTGAGATGATGCTTTGAC-3′	481–505		
IL-4	NM_000589	sense	5′-GTCCACGGACACAAGTGCGAT-3′	129–139	369 bp	(1)
		antisense	5′-CATGATCGTCTTTAGCCTTTC-3′	477–497		
IL-5	J03478	sense	5′-GCTTCTGCATTTGAGTTTGCTAGCT-3′	9–33	354 bp	(1)
		antisense	5′-TGGCCGTCAATGTATTTCTTTATTAAG-3′	276–362		
IL-6	AF372214	sense	5′-ATGAACTCCTTCTCCACAAGCGC-3′	1–23	628 bp	(1)
		antisense	5′-GAAGAGCCCTCAGGCTGGACTG-3′	607–628		
IL-10	M57627	sense	5′-ATGCCCCAAGCTGAGAACCAAGACCCA-3′	313–339	355 bp	(1)
		antisense	5′-GTTTCTCAAGGGGCTGGGTCAGCTATCCCA-3′	638–667		
TNF-α	M10988	sense	5′-GACCTCTCTCTAATCAGCCC-3′	281–300	304 bp	(1)
		antisense	5′-CCTTGGTCTGGTAGGAGACG-3′	565–584		
IFN-γ	AF375790	sense	5′-ATGAAATATACAAGTTATATCTTGGCTTT-3′	1–29	494 bp	(1)
		antisense	5′-GATGCTCTTCGACCTCGAAACAGCAT-3′	469–494		
Chemokines						
Mig	14727465	sense	5′-TTCCTCTTGGGCATCATCTT-3′	22–41	265 bp	(2)
		antisense	5′-TTTGGCTGACCTGTTTCTCC-3′	267–286		
IP-10	NM_001565	sense	5′-GGAACCTCCAGTCTCAGCACCA-3′	46–67	245 bp	(2)
		antisense	5′-AGACATCTCTTCTCACCCTTC-3′	270–290		

Coagulation-related proteins						
TF	J02931	sense	5′-CTACTGTTTCAGTGTTCAAGCAGTGA-3′	759–784	283 bp	(3)
		antisense	5′-CAGTGCAATATAGCATTTGCAGTAGC-3′	1016–1041		
PAI-1	X04744	sense	5′-GTGTTTCAGCAGGTGGCGC-3′	76–94	300 bp	(3)
		antisense	5′-CCGGAACAGCCTGAAGAAGTG-3′	351–375		
chemokine receptor						
CXCR3	X95876	sense	5′-CCACTGCCAATACAACTTCC-3′	671–690	401 bp	(4)
		antisense	5′-GCAAGAGCAGCATCCACATC-3′	1052–1071		
growth factor						
VEGF	XM_052678	sense	5′-TCGGGCCTCCGAAACCATG-3′	23–41	517/649 bp	(4)
		antisense	5′-CCTGGTGAGAGATCTGGTT-3′	653–671		
Influenza A virus protein						
M2	M63515	sense	5′-ATGAGCCTTCTAACCGAGGT-3′	26–45	294/982 bp	(1)
		antisense	5′-TTACTCCAGCTCTATGCTGACA-3′	986–1007		
Structure protein						
β-actin	M10277	sense	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′	468–497	661 bp	(1)
		antisense	5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′	1099–1128		

(1) 35 cycles consisting of 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C
(2) 35 cycles consisting of 0.5 min at 94 °C, 1 min at 63 °C, and 1 min at 72 °C
(3) 35 cycles consisting of 1 min at 94 °C, 1.25 min at 55 °C, and 1.25 min at 72 °C
(4) 35 cycles consisting of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C

Real-time PCR

Real-time PCR was performed using the SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA) according to manufacturer's protocol. Following primer sequences were used: IP-10 forward (AGCCAATTTTGTCCACGTGTT), reverse (GGCCTTC GATTCTGGATTCAG); Mig forward (TCTTTTCCTCTTGGGCATCATC), reverse (CAGG AACAGCGACCCTTTCTC); β-actin forward (CCTGGCACCCAGCACAAT), reverse (GC CGATCCACACGGAGTACT). One μl of cDNA described above was used for the 100-μl reaction solution. The following conditions of ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) were used: Initial denaturation at 94 °C for 10 min, followed by 45 cycles with denaturation at 94 °C for 20 sec, annealing and elongation at 60 °C for 1 min. Quantities of specific mRNA in the sample were measured according to the corresponding gene-specific standard curve. The level of expression of IP-10 and Mig gene were normalized to that of β-actin gene.

Sequencing

The PCR-amplified products were sequenced directly by using an ABI PRISM^{TM} Dye terminator cycle sequencing ready reaction kit with an ABI 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA).

Results

HUVECs are susceptible to Influenza A virus *infection*

To determine the susceptibility of HUVECs to *Influenza A virus* infection, influenza A/Aichi/2/68 (H3N2) virus propagated in MDCK cells was inoculated onto HUVECs. Cells were harvested at 24 h post infection (p.i.), and viral proteins were analyzed by Western blot. The specific reactivity of the antiserum to the components of influenza A/Aichi/2/68 (H3N2) has been determined (data not shown). A 30-kDa protein, most likely matrix protein (M1) was detected in the infected HUVECs (Fig. 1A, lane 1, asterisk), whereas this protein was not found in uninfected (Fig. 1A, lane 2), mock-infected (Fig. 1A, lane 3) and heat-inactivated virus-inoculated HUVECs (Fig. 1A, lane 4). Although the sizes of 60-kDa and 97-kDa bands detected in the infected HUVECs (Fig. 1A, lane 1) were consistent with those of nucleoprotein (NP) and uncleaved HA respectively, this analysis could not specify these viral proteins. The viral 60-kDa band was overlapped with a cellular 60-kDa protein, which was reacted with the chicken polyclonal antiserum to *Influenza A virus* (Fig. 1A, lane 2). To determine whether these bands are viral proteins, we then used mouse monoclonal antibody specific to HA. Uncleaved HA was detected in the infected (Fig. 1B, lane 2), but not in mock-infected HUVECs (Fig. 1B, lane 1). These results demonstrated that viral protein synthesis occurred in the infected HUVECs.

The expression of viral proteins in HUVECs was also analyzed at 1, 5, 10 and 24 h p.i. and compared to that of mock infection (Fig. 1C). The viral proteins became detectable at 10 h p.i., and the level of protein expression rose at 24 h p.i. (Fig. 1C).

The M2 protein is assumed to function as a pH-activated ion channel and is expressed on the surface of virus-infected cells [10]. The M2 protein is produced

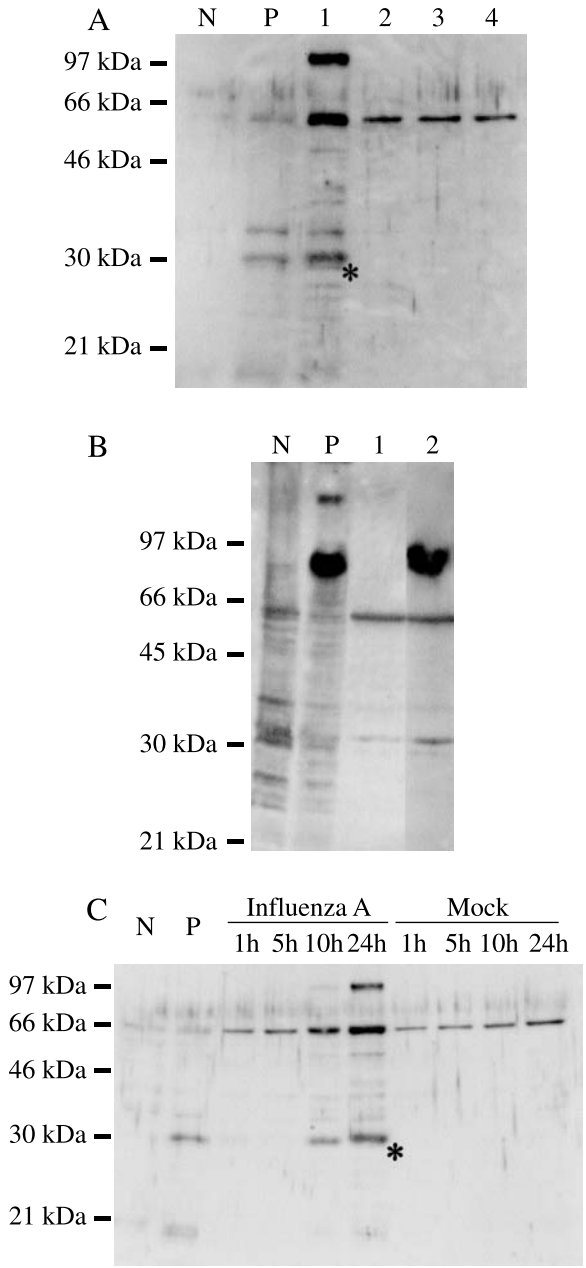

Fig. 1. A Western blot analysis in HUVECs infected with *Influenza A virus* using chicken polyclonal antiserum to influenza A/Aichi/2/68 (H3N2). Influenza A virus-infected HUVECs (*1*), uninfected HUVECs (*2*), mock-infected HUVECs (*3*) and heat-inactivated virus-inoculated HUVECs (*4*). **B** Western blot analysis in HUVECs infected with *Influenza A virus* using mouse monoclonal antibody (L164/1) specific to HA. Influenza A virus-infected HUVECs (*2*), mock-infected HUVECs (*1*). **C** Time course of viral protein expression in HUVECs after infection of *Influenza A virus*. Total protein was harvested at different time points (1, 5, 10 and 24 h after infection) from infected and mock-infected HUVECs. Asterisk showed the viral structual proteins: matrix protein 1 (M1)(*). Uninfected MDCK cells (*N*) and *Influenza A virus*-infected MDCK cells (*P*) were used as negative and positive controls, respectively

in infected cells by alternative splicing of RNA segment 7 of *Influenza A virus* [10]. The cDNA was synthesized from HUVECs infected with *Influenza A virus* and amplified using M2-1 and M2-2 primers shown in Fig. 2A. Bands of 982- and 294-bp were detected from infected HUVECs (Fig. 2B, lanes 4–7) and were confirmed by sequencing to be parts of RNA segment 7 and the M2 gene of influenza A/Aichi/2/68, respectively (data not shown) [22]. The M2-specific 294-bp product was not amplified at 1 h p.i. (Fig. 2B, lane 4) but was detected at 5 h

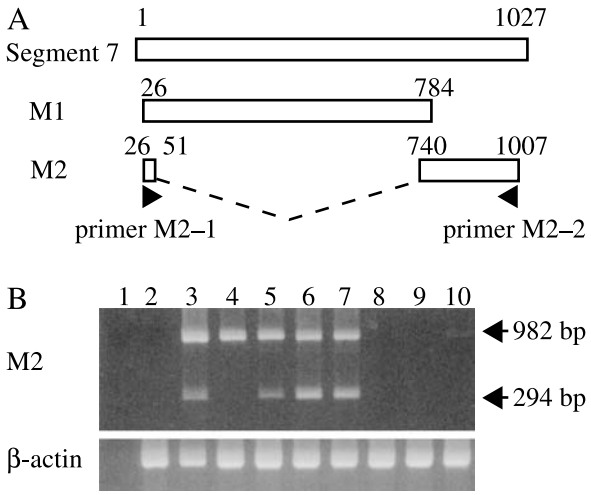

Fig. 2. **A** Design of PCR primers. The M1 and M2 proteins of *Influenza A virus* were produced by alternative splicing of RNA segment 7. Small arrows indicated the PCR primers (M2-1 and M2-2) used for amplification. The 982- and 294-bp products were parts of RNA segment 7 and the M2 gene of influenza A/Aichi/2/68, respectively. **B** Detection of M2-specific products in RT-PCR. *1*, negative control (double-distilled water, DDW); *2*, negative control (MDCK cells); *3*, positive control (infected MDCK cells); *4–7*, infected HUVECs, at 1, 5, 10 and 24 h after infection; *8*, HUVECs; *9*, mock infection (24 h after infection); *10*, heat-inactivated virus infection (24 h after infection)

p.i. (Fig. 2B, lane 5), demonstrating that the virus RNA was transcribed in the infected HUVECs. The maximum amplification of mRNA was seen at 10 h p.i. (Fig. 2B, lane 6). The M2-specific RT-PCR product was not detected from the purified virus itself (data not shown). The results of Western blot analysis and RT-PCR demonstrated that HUVECs are susceptible to *Influenza A virus* infection and allow transcription and translation of the viral genes.

Levels of IP-10, Mig and IL-6 gene expression are elevated in infected HUVECs

We next examined the profiles of cytokine and chemokine gene expressions in HUVECs infected with *Influenza A virus*. Total RNA was extracted at 5 h p.i. and analyzed by the RT-PCR method. Table 1 shows the primer pairs and the PCR conditions of each amplification: 8 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, TNF-α and INF-γ), 2 chemokines (Mig and IP-10), 2 coagulation-related proteins (tissue factor, TF, and plasminogen activator inhibitor-1, PAI-1), a chemokine receptor (CXCR3), a growth factor (vascular endothelial growth factor, VEGF), the M2 protein of *Influenza A virus* and a cell structural protein gene (β-actin). Representative results of PCR are shown in Fig. 3. Each experiment was repeated at least 3 times. In these experiments using conventional RT-PCR method, the expression levels of four genes (IP-10, Mig, IL-6 and M2) were elevated in response to *Influenza A virus* infection in

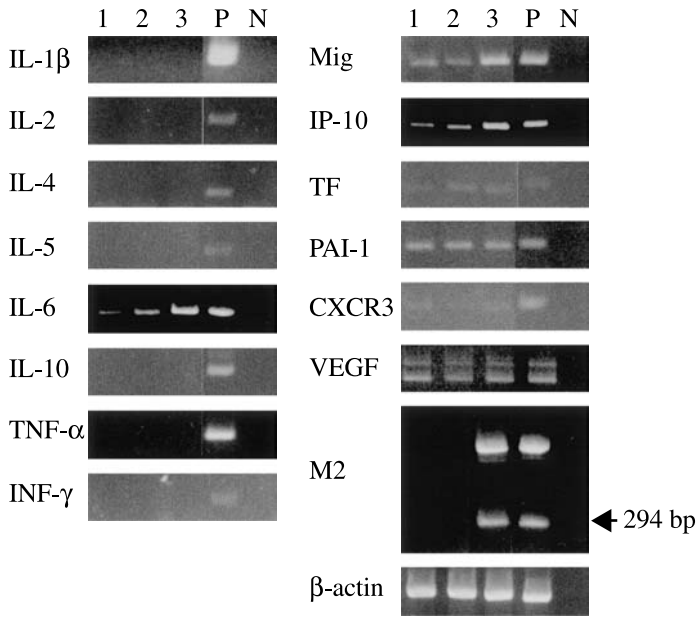

Fig. 3. Profiles of gene expression in *Influenza A virus* infected-HUVECs determined by RT-PCR. HUVECs were infected with *Influenza A virus*. Total RNA was harvested at 5 h after infection. The primers and conditions of the PCR reaction used are shown in Table 1. *1*, HUVECs; *2*, mock-infected HUVECs; *3*, *Influenza A virus*-infected HUVECs; *P*, positive control (peripheral blood mononuclear cells stimulated by phytohemagglutinin for 8 cytokines, *VEGF* and *β-actin*; spleen cells for Mig, *IP-10* and *CXCR3*; HUVECs for TF and *PAI-1*; influenza A virus-infected MDCK for M2); *N*, negative control (DDW)

HUVECs. The M2-specific 294-bp product was amplified as a result of *Influenza A virus* infection in HUVECs as previously described (Fig. 2A, 2B). To assess the relative quantity of IP-10 and Mig in the infected HUVECs, the expression levels of IP-10, Mig and β-actin were measured by real-time PCR. Measurements were performed in triplicates. The relative quantities of IP-10 and Mig to HUVECs were shown in Fig. 4. The expression levels of IP-10 and Mig in infected HUVECs were increased to 24.5 and 52 folds, but not in mock-infected cells.

In the present study, we focused on the elevation of chemokine gene expression, which has not been described previously. To confirm the elevation of IP-10 and Mig gene expression, we analyzed the gene expression in HUVECs infected with *Influenza A virus* at different time points. As shown in Fig. 5, *Influenza A virus* infection induced a time-dependent elevation of these gene expression in HUVECs compared to that in the control. The signal increased at 5 h p.i. and its expression remained almost constant to 24 h p.i. CXCR3 has been cloned and characterized as a specific receptor of the CXC chemokines IP-10 and Mig [30]. Increase in CXCR3 gene expression was not observed in the *Influenza A virus*-infected HUVECs by conventional RT-PCR method (Fig. 3) [17].

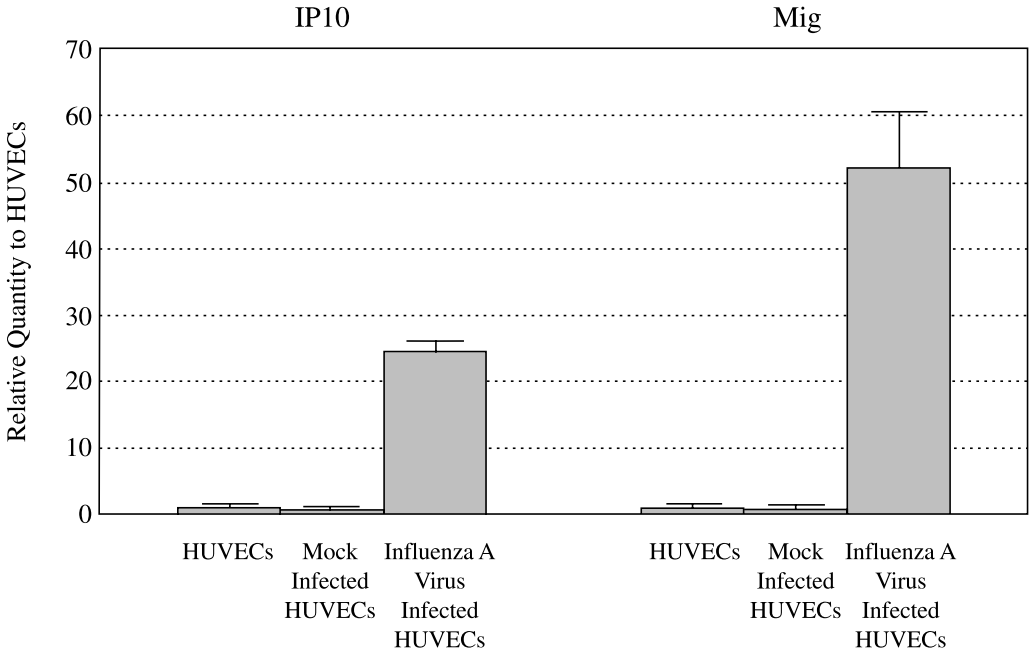

Fig. 4. Summary of IP-10 and Mig mRNA expression in *Influenza A virus* infected HUVECs. Bar graphs represent mRNA expression of IP-10 and Mig in mock-infected and *Influenza A virus*-infected HUVECs compared to control HUVECs. Data are given as means ± SEM

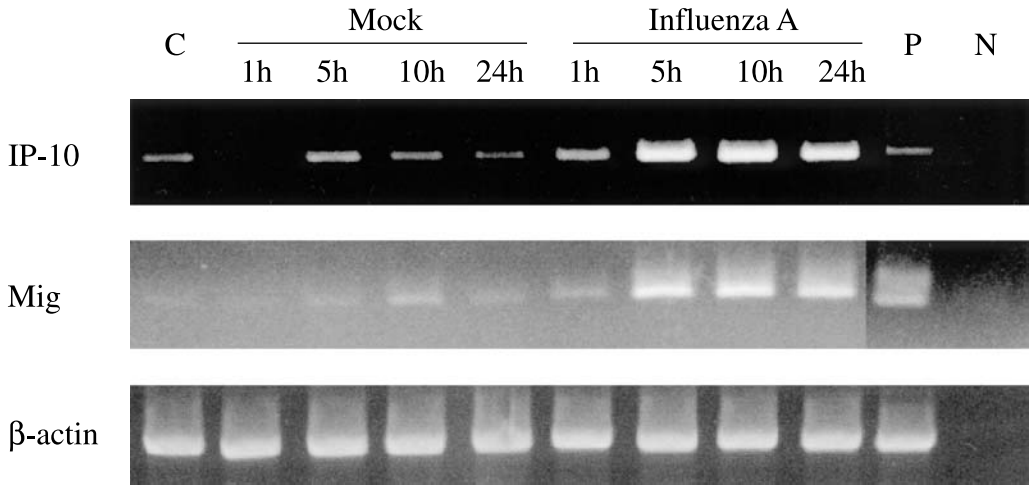

Fig. 5. Analysis by RT-PCR of IP-10 and Mig gene expression in HUVECs infected with *Influenza A virus*. After HUVECs were infected with *Influenza A virus*, total RNA was harvested at different time points (1, 5, 10 and 24 h after infection). The IP-10 and Mig gene expression in infected HUVECs (*Influenza A*) was compared to that in mock-infected HUVECs (*Mock*) and uninfected HUVECs (*C*). DDW (*N*) and spleen cells (*P*) were used as negative and positive controls, respectively

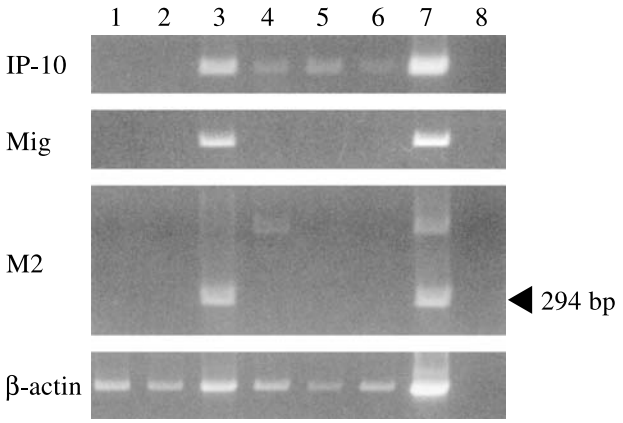

Fig. 6. Analysis of IP-10 and Mig gene expression in HUVECs infected with purified *Influenza A virus* by RT-PCR. HUVECs were inoculated with only MEM (*2*) or infected with live purified (*3*), heat-inactivated (*4*), formalin-inactivated (*5*) and diethyl ether-inactivated *Influenza A virus* (*6*) at an MOI of 1. HUVECs were incubated for an additional 10 h, and the IP-10 and Mig gene expression was analyzed. *1*, HUVECs; *7*, positive control (spleen cells for IP-10, Mig and β-actin; *Influenza A virus*-infected MDCK for M2); *8*, negative control (DDW)

Live virus infection is required for full elevation of chemokine gene expression in HUVECs

To investigate the potential of inactivated *Influenza A virus* to elevate chemokine gene expression, HUVECs were infected with purified live *Influenza A virus* at an MOI of 1 or inoculated with an equivalent amount of inactivated *Influenza A virus* for 1 h at 37 °C, and the cells were then cultured for an additional 10 h. The levels of IP-10 and Mig gene expression were analyzed by RT-PCR. The results are shown in Fig. 6. The IP-10 gene expression was slightly elevated in HUVECs inoculated with heat-, formalin- or diethyl ether-inactivated viruses compared to that in HUVECs infected with purified live virus (Fig. 6, lanes 4–6). The Mig gene expression was not elevated in any of the HUVECs inoculated with inactivated viruses. The fact that no M2-specific RT-PCR products were detected indicates that each of the inactivation procedures leaded complete loss of infectivity of the virus.

CHX does not inhibit the elevation of IP-10 and Mig gene expression by infection with Influenza A virus

To determine whether the elevation of chemokine gene expression requires viral and/or host protein synthesis, HUVECs were treated with CHX (an inhibitor of protein synthesis) for 60 min prior to infection. Incubation of HUVECs with 10 μg/ml CHX did not cause death of HUVECs (data not shown). After infection, the HUVECs were incubated for additional 10 h in the HUVEC's medium with 10 μg/ml CHX. The levels of IP-10 and Mig gene expression were analyzed by RT-PCR. The levels of IP-10 and Mig gene expression in the HUVECs after

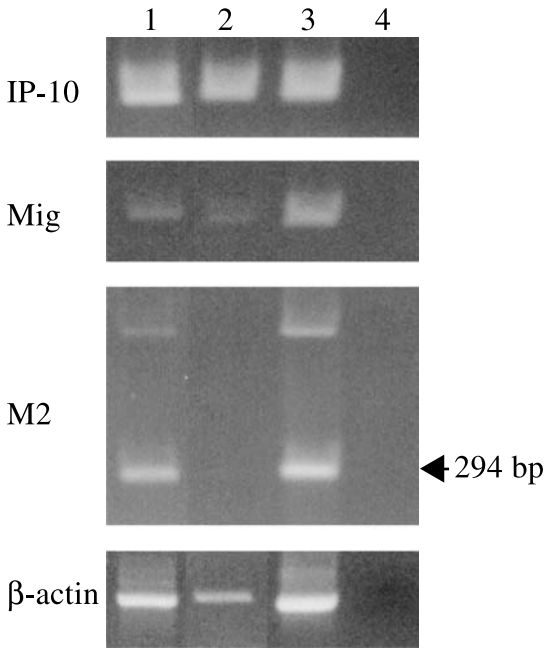

Fig. 7. Effect of cycloheximide on the IP-10 and Mig gene expression. HUVECs were treated with CHX, and infected with *Influenza A virus* with (*2*) or without (*1*) CHX. The expression of IP-10, Mig, M2 and β-actin genes was analyzed by RT-PCR. *3*, positive control (spleen cells); *4*, negative control (DDW)

Influenza A virus infection with CHX were similar to those without CHX (Fig. 7, lanes 1 and 2). These suggest that the process of elevation of IP-10 and Mig gene expression in HUVECs upon infection with *Influenza A virus* does not require new protein synthesis. On the other hand, the M2-specific 294-bp product was drastically suppressed in the HUVECs after *Influenza A virus* infection with CHX (Fig. 7, lane 2). These results suggest that CHX successfully inhibited the protein synthesis of HUVECs, because the synthesis of M2 protein in *Influenza A virus*-infected cells requires the translation of polymerase complexes (PA, PB1 and PB2) [10].

Discussion

In the present study, we first demonstrated that *Influenza A virus* infects in human endothelial cells by Western blot analysis (Fig. 1A–C) and by RT-PCR (figure 2B). Influenza viruses are known to infect and replicate in bronchial epithelial cells [13, 43]. On the other hand, influenza virus infects but seldom replicates in non-respiratory tissues [11, 42]. Certain strains of avian influenza virus fully replicate in chicken vascular endothelial cells [24]. To determine whether infected HUVECs produce viral particles, we measured the virus titers of the culture supernatants collected from infected HUVECs using MDCK cells in three different series of experiments. In spite of repetitive tests, we could not detect any significant increase in the virus titer at 24 and 48 h p.i. (data not shown). These results suggest that *Influenza A virus* infection is abortive in HUVECs or that the production of infectious virions is limited.

A significant finding is that *Influenza A virus* infection induced elevation of IP-10 and Mig gene expression in endothelial cells (Figs. 3 and 4), whereas the basal expression of two genes in uninfected HUVECs were undetectable or minimum if any (Fig. 3, lane 1; Fig. 5, lane C; Fig. 6, lane 1 and 2). In every experiment, there was definite elevation in IP-10 and Mig gene expression in infected HUVECs compared to that in mock-infected HUVECs (Figs. 3, 4, 5 and 6; data not shown). The elevation in IP-10 and Mig gene expression was also confirmed by the time courses experiment (Fig. 5) and by the experiment using purified virus (Fig. 6). HUVECs infected with mock virus did not have any positive message of IP-10 at 1 hr incubation in this experiment (Fig. 5). In another series of experiments, IP-10 signal in mock-infected HUVECs was consistently detected at any time point. Although IL-6 induction in HUVECs by *Influenza A virus* was described previously [59], this was the first report of elevation of IP-10 and Mig gene expression in human endothelial cells infected with *Influenza A virus*. IP-10 and Mig are members of the CXC subfamily of chemokines [40] and were originally characterized as early response genes induced after IFN-γ treatment in a variety of cells [46]. IP-10 can also be induced by IFN-α and IFN-β as well as by lipopolysacharide and some other proinflammatory cytokines, such as TNF-α [38, 39]. We therefore examined the levels of IFN-γ and TNF-α gene expression in *Influenza A virus*-infected HUVECs in parallel with those of IP-10 and Mig gene expression. There was no significant increase in the levels of IFN-γ and TNF-α gene expression at any time point examined (Fig. 3, time-course data not shown). Influenza virus infection in HUVECs with CHX resulted in the elevation of IP-10 and Mig gene expression (Fig. 7, lane 2), which was comparable to that without CHX (Fig. 7, lane 1). These data support that the process of elevation of IP-10 and Mig gene expression in HUVECs infected with *Influenza A virus* does not require new protein synthesis and be independent from INF-γ pathway. Recently the IFN-independent pathways of IP-10 induction has been reported in other assay systems [2, 26]. From the results of the experiments using inactivated viruses (Fig. 6), it is apparent that full elevation of IP-10 and Mig gene expression requires virus infection.

To address the question whether the elevation of IP-10 and Mig expression upon *Influenza A virus* infection is a specific phenomenon of A/Aichi/2/68 strain, A/PR/8/34 strain was inoculated to HUVECs. The M2-specific 294-bp product was amplified (data not shown), demonstrating that A/PR/8/34 strain could also infect to HUVECs. Increase of IP-10 and Mig gene expression was observed at 10 h p.i., and became prominent at 24 h p.i. (data not shown). Thus, the elevation of IP-10 and Mig gene expression were not a specific phenomenon of Influenza A/Aichi/2/68 strain.

IL-6 is a multifunctional cytokine, and may also influence leukocyte recruitment [21]. However IL-6 itself had little or no effect on IP-10 expression in primary human astrocytes [37], astroglioma cell line [37], HUVECs [44] and peritoneal mesothelial cells [21]. Thus elevated gene expression of IP-10 after *Influenza A virus* infection in our experiments was supposed to be regulated by different mechanism from IL-6 systems.

IP-10 is produced from endothelial cells in response to various virus infections, e.g., hantavirus and mouse adenovirus-type 1 infections [8, 55]. Infection by *Borrelia burgdorferi*, the causative protozoan of Lyme disease, elevates the Mig gene expression in endothelial cells [12]. IP-10 and Mig have been shown to exhibit direct antiviral properties [31, 33, 48] and to be essential in the development of a protective Th1 response against viral infection in the central nervous system [28, 29]. Both IP-10 and Mig have been reported to play roles in the pathogenesis of tissue necrosis and vascular damage associated with certain EBV-positive lymphoproliferative processes [57]. It is still not clear whether influenza virus infection *in vivo* is actually associated with elevation of IP-10 and Mig gene expression in endothelial cells. In our preliminary experiments, the expression of both chemokine genes were greatly elevated in the cerebrum of an autopsy case of influenza encephalitis (data not shown). We could not generalize our findings in overall pathogenesis of influenza virus infection in humans, because endothelial cells are not primary target cells of human influenza A viruses. However IP-10 and Mig might play some roles in the pathogenesis of vascular damage and blood-brain barrier destruction in some limited cases like influenza encephalitis/ encephalopathy.

It has recently been reported that members of the *Herpesviridae* family (cytomegalovirus and herpes simplex virus) can damage endothelial cells. Herpes virus infection of vascular endothelial cells can alter the normal thrombo-resistant surface and alter hemostasis by increasing amount of plasminogen activator inhibitor type 1 (PAI-1) and tissue factor (TF) [19]. We did not detect a significant change in PAI-1 and TF gene expression in infected HUVECs by conventional RT-PCR method (Fig. 3). However further studies using real-time quantitative PCR will be necessary to conclude the roles of coagulation factors in vascular damage of *Influenza A virus*.

In summary, the present study demonstrated that *Influenza A virus* infection elevated the of IP-10 and Mig gene expression in HUVECs. The elevation of IP-10 and Mig gene expression after *Influenza A virus* infection was independent of the IFN-γ pathway.

Acknowledgments

The authors thank Mr. Stewart Chisholm for proofreading the manuscript.

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