Sakoda, Y.et al.	Reintroduction of H5N1 highly pathogenic avian influenza virus by migratory water birds, causing poultry outbreaks in 2010-2011 winter season in Japan.	J Gen Virol	93	541-550	2012
Nomura, N.et al.	An H9N2 influenza virus vaccine prepared from a non-pathogenic isolate from a migratory duck confers protective immunity in mice against challenge with an H9N2 virus isolated from a girl in Hong Kong.	J Vet Med Sci	71	441-447	2012
Nomura, N.et al.	Characterization of avian influenza viruses isolated from domestic ducks in Vietnam in 2009 and 2010.	Arch Virol	157	247-257	2012
Arikata, M.et al.	Memory immune responses against pandemic (H1N1) 2009 influenza virus induced by a whole particle vaccine in cynomolgus monkeys carrying Mafa-A1*052ratio02	PLoSOne	7	E37220	2012
Isoda, N. et al.	The nucleoprotein is responsible for interacerebral pathogenicity of A/duck/Mongolia/47/2001 (H7N1) in chicks.	Arch Virol	157	2257-2264	2012
Kajihara, M.et al.	The PB2, PA, HA, NP, and NS genes of a highly pathogenic avian influenza virus A/whooper swan/Mongolia/3/2005 (H5N1) are responsible for pathogenicity in ducks.	Virol J	10	45	2013
Okamatsu, M.et al.	Potency of a vaccine prepared from A/swin e/Hokkaido/2/1981 (H1N1) against A/Narita/1/2009 (H1N1) pandemic influenza virus strain.	Virol J	10	47	2013

Yamamoto, N.et al.	Proteins of duck influenza virus responsible for acquisition of pathogenicity in chickens.	Virus Res	In press		
Shichinohe, S.et al.	Potency of an inactivated influenza vaccine prepared from a non-pathogenic H5N1 virus against a challenge with antigenically drifted highly pathogenic avian influenza viruses in chickens.	Vet Microbiol	164	39-45	2013
Nakajima N, et al.	Pathological study of archival lung tissues from five fatal cases of avian H5N1 influenza in Vietnam.Mod	Pathol. Epub	10.103	193	2012
Sasaki I, et al.	Spi-B is critical for plasmacytoid dendritic cell function and development	Blood Epub			2012
van Riet E, et al.	Mucosal IgA responses in influenza virus infections; thoughts for vaccine design.	Vaccine Epub	31;30(40)	5893-900	2012
Hamada N, et al.	Intrahost emergent dynamics of oseltamivir-resistant virus of pandemic influenza A (H1N1) 2009 in a fatally immune- compromised patient.	J Infect Chemother	18	865-871	2012
Nakazono Y, et al.	The RNA polymerase PB2 subunit of influenza A/HongKong/156/1997(H5N1) restricts the replication of reassortant ribonucleoprotein complexes.	PLoS ONE	7(2)	e32634	2012
Kenri T, et al.	Identification of Mycoplasma pneumoniae type 2b variant strains in Japan.	J. Med. Microbiol.	61(Pt 11)	1633-5	2012

鈴木里和、他	2011年流行時における Mycoplasma pneumoniae 感染症による入院患者の臨床的検討	IASR	33	162-163	2012
Miyasaka T, et al.	A possible relationship of natural killer T cells with humoral immune response to 23-valent pneumococcal polysaccharide vaccine in clinical settings.	Vaccine	33	3304-10	2012
Oishi T, et al.	Low opsonic activity to the infecting serotype in pediatric patients with invasive pneumococcal disease.	Vaccines	31	845-9	2013
Mori S, et al.	Impact of tocilizumab therapy on antibody response to influenza vaccine in patients with rheumatoid arthritis.	Ann Rheum Dis	71	2006-10	2012
Mori S, et al	Pneumococcal polysaccharide vaccination in rheumatoid arthritis patients receiving tocilizumab therapy.	Ann Rheum Dis	Jan 23. [Epub ahead of print]		2013
Takei S, Hotomi M, Yamanaka N.	Minimal biofilm eradication concentration of antimicrobial agents against nontypeable Haemophilus influenzae isolated from middle ear fluids of intractable acute otitis media	J Infect Chemother	In press		2013
Hotomi M, et al.	PspA family distribution, antimicrobial resistance and serotype of Streptococcus pneumoniae isolated from upper respiratory tract infections in Japan	PLoS One	8(3)		2013

Hotomi M, et al.	Evaluation of a rapid immunochromatographic ODK-0901 test for detection of pneumococcal antigen in middle ear fluids and nasopharyngeal secretions	PLoS One	In press		2013
Tanaka J, et al.	Incidence of childhood pneumonia and serotype and sequence-type distribution in Streptococcus pneumoniae isolates in Japan.	Epidemiol Infect.	140	1111-21	2012
Umeyama T, et al.	Determination of epidemiology of clinically isolated Cryptococcus neoformans strains in Japan by multilocus sequence typing.	Jpn J Infect Dis	66	51-55	2013
二木 芳人、他	専門医部会 成人予防接種のガイダンス	日本内科学会雑誌	101	3585-3597	2012
Qin L, et al.	Impaired capsular polysaccharide is relevant to enhanced biofilm formation and lower virulence in Streptococcus pneumoniae	J Infect Chemother	Dec 11	[Epub ahead of print]	2012
Hamada N, et al.	Intrahost emergent dynamics of oseltamivir-resistant virus of pandemic influenza A (H1N1) 2009 in a fatally immunocompromised patient.	J Infect Chemother	18	865-871	2012

Nakazono Y, et al.	The RNA polymerase PB2 subunit of influenza A/HongKong/156/1997(H5N 1) restricts the replication of reassortant ribonucleoprotein complexes.	PLoS ONE	7(2)	e32634	2012
Qin L, et al.	Antimicrobial susceptibilities and genetic characteristics of Haemophilus influenzae isolated from community acquired respiratory tract infection patients in Shanghai City, China	J Infect Chemother	18	508-514	2012
Toyotome T, et al.	Fetuin A, a serum component, promotes growth and biofilm formation by Aspergillus fumigatus	Int J Med Microbiol	302	108-116	2012

Ⅳ 研究成果の刊行物・別刷

Redox-Active Protein Thioredoxin-1 Administration Ameliorates Influenza A Virus (H1N1)-Induced Acute Lung Injury in Mice

Masato Yashiro, MD[1]; Hirokazu Tsukahara, MD, PhD[1]; Akihiro Matsukawa, MD, PhD[2]; Mutsuko Yamada, BS[1]; Yosuke Fujii, MD[1]; Yoshiharu Nagaoka, MD[1]; Mitsuru Tsuge, MD, PhD[1]; Nobuko Yamashita, MD, PhD[1]; Toshihiro Ito, MD, PhD[2]; Masao Yamada, MD, PhD[3]; Hiroshi Masutani, MD, PhD[4]; Junji Yodoi, MD, PhD[4,5]; Tsuneo Morishima, MD, PhD[1]

Objectives: Influenza virus infections can cause severe acute lung injury leading to significant morbidity and mortality. Thioredoxin-1 is a redox-active defensive protein induced in response to stress conditions. Animal experiments have revealed that thioredoxin-1 has protective effects against various severe disorders. This study was undertaken to evaluate the protective effects of recombinant human thioredoxin-1 administration on influenza A virus (H1N1)-induced acute lung injury in mice.

Design: Prospective animal trial.

Setting: Research laboratory.

Subjects: Nine-week-old male C57BL/6 mice inoculated with H1N1.

Intervention: The mice were divided into a vehicle-treated group and recombinant human thioredoxin-1-treated group. For survival rate analysis, the vehicle or recombinant human thioredoxin-1 was administered intraperitoneally every second day from day −1 to day 13. For lung lavage and pathological analyses, vehicle or recombinant human thioredoxin-1 was administered intraperitoneally on days −1, 1, and 3.

Measurements and Main Results: Lung lavage and pathological analyses were performed at 24, 72, and 120 hrs after inoculation. The recombinant human thioredoxin-1 treatment significantly improved the survival rate of H1N1-inoculated mice, although the treatment did not affect virus propagation in the lung. The treatment significantly attenuated the histological changes and neutrophil infiltration in the lung of H1N1-inoculated mice. The treatment significantly attenuated the production of tumor necrosis factor-α and chemokine (C-X-C motif) ligand 1 in the lung and oxidative stress enhancement, which were observed in H1N1-inoculated mice. H1N1 induced expressions of tumor necrosis factor-α and chemokine (C-X-C motif) ligand 1 in murine lung epithelial cells MLE-12, which were inhibited by the addition of recombinant human thioredoxin-1. The recombinant human thioredoxin-1 treatment started 30 mins after H1N1 inoculation also significantly improved the survival of the mice.

Conclusions: Exogenous administration of recombinant human thioredoxin-1 significantly improved the survival rate and attenuated lung histological changes in the murine model of influenza pneumonia. The protective mechanism of thioredoxin-1 might be explained by its potent antioxidative and anti-inflammatory actions. Consequently, recombinant human thioredoxin-1 might be a possible pharmacological strategy for severe influenza virus infection in humans. (*Crit Care Med* 2013; 41:171–181)

Key Words: acute lung injury; cytokine; influenza virus; mouse; oxidative stress; thioredoxin-1

[1] Department of Pediatrics, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.
[2] Department of Pathology and Experimental Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.
[3] Department of Virology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.
[4] Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto, Japan.
[5] Center for Cell Signaling Research and Department of Bioinspired Science, Ewha Womans University, Seoul, Korea.

This work was supported by research grants from the Japanese Ministry of Health, Labour and Welfare (Dr. Morishima), the Japanese Ministry of Education, Culture, Sports, Science and Technology (Dr. Morishima), and the World Class University Grant R31-10010 (Dr. Yodoi).

The authors have not disclosed any potential conflicts of interest.

For information regarding this article, E-mail: tsukah-h@cc.okayama-u.ac.jp

Influenza virus infections cause a broad array of illnesses that are responsible for significant morbidity and mortality both in children and adults on a yearly basis (1). Influenza can cause periodic global pandemics with even higher penetrance of illness. Highly pathogenic avian influenza virus H5N1 emerged in 1996 in Hong Kong, China (2). Although cases of avian influenza infections have decreased since 2006, the emergence of a pandemic strain remains a threat. In 2009, novel swine-origin influenza virus H1N1 was identified in Mexico. It continues to spread globally (3).

DOI: 10.1097/CCM.0b013e31826be81d

Several antiviral compounds have been developed against influenza virus to interfere with specific events in the replication cycle. Under treatment with these drugs, however, influenza virus infection occasionally causes severe pneumonia, necessitating intensive care and mechanical ventilation in the clinical settings. The course of illness and complications might also be affected by coexisting pathologies. The discovery of a novel anti-influenza therapeutic approach would increase the effectiveness of traditional virus-based strategies (4).

Reactive oxygen species (ROS) such as superoxide, hydroxyl radical, and nitric oxide represent an important component of the host's arsenal to combat invading micro-organisms, but ROS present significant immunopathology to surrounding tissues because of their toxicity and lack of specificity (5, 6). It is recognized that much of the oxidative injury associated with simultaneous production of superoxide and nitric oxide is mediated by a strong oxidant: peroxynitrite (7). The pathogenesis of influenza pneumonia is almost certain to involve not only apoptotic cell death mediated through viral replication in the infected cells, but also the injury of noninfected cells by ROS derived from infiltrating neutrophils and macrophages and respiratory tract epithelium (4). A critical role of ROS as mediators of influenza virus-induced lung injury is supported by previous studies. In murine models of influenza pneumonia, excessive generation of ROS contributed to lung injury in infected animals; treatment with superoxide dismutase, catalase (antioxidative enzymes), N-monomethyl-L-arginine (nitric oxide synthase inhibitor), or allopurinol (xanthine oxidase inhibitor), and overexpression of extracellular superoxide dismutase or heme oxygenase-1 suppressed lung injury and inflammation and improved the survival rate (8–13). Furthermore, virus-infected selenium-deficient mice developed more severe influenza pneumonia than did selenium-adequate mice, implying the importance of selenium-dependent glutathione peroxidase and thioredoxin reductase (antioxidative enzymes) for protection against influenza virus-induced inflammatory processes (14).

Thioredoxin-1 (TRX-1), a redox-active small protein that is ubiquitous in the body, is a defensive protein that is induced in response to various stress conditions (15). In addition to its antioxidative effect by dithiol–disulfide exchange in its active site, TRX-1 has anti-inflammatory and antiapoptotic effects (15, 16). Human TRX-1-overexpressing transgenic mice survive longer and are more resistant to various oxidative and inflammatory conditions than control mice (15, 17). Of greater importance is the fact that TRX-1 overexpression is also effective in augmenting the host defense against influenza pneumonia, thereby reducing mortality (18). Overexpression of TRX-1 might modulate the ROS generation induced by influenza virus infection and regulate the redox-dependent signal transductions in the host defense responses against influenza virus infection.

These findings prompted us to evaluate the protective effects of recombinant human TRX-1 (rhTRX-1) administration on acute lung injury in mice with influenza pneumonia. If rhTRX-1 administration can produce significant beneficial effects, then its clinical efficacy for treating severe pneumonia will be highly anticipated. This study demonstrated for the first time that rhTRX-1 administration decreases the mortality rate and ameliorates acute lung injury in influenza A virus (H1N1)-infected mice, possibly through its antioxidative and anti-inflammatory actions.

MATERIALS AND METHODS

This study was approved by the Animal Use Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and was conducted in accordance with National Institutes of Health Guidelines.

Experimental Animals

Eight-week-old male C57BL/6 mice (21–24 g body weight) were purchased from Charles River Laboratories (Yokohama, Japan). They were housed in the specific-pathogen-free animal facility at 25°C with a 12-hr light/dark cycle. They were fed a standard diet (Oriental MF; Oriental Yeast, Ltd., Tokyo, Japan).

Regional Distribution of TRX-1 mRNA

Nine-week-old mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and pentobarbital (30 mg/kg). The brain, lung, liver, kidney, and spleen were removed and soaked in RNAlater (Applied Biosystems, Foster City, CA). Total RNA was extracted using RNeasy Plus Mini (Qiagen, Hilden, Germany). Two micrograms of total RNA was reverse-transcribed to cDNA using RETROscript (Applied Biosystems). Reverse-transcribed samples were analyzed for murine TRX-1 and glyceraldehyde-3-phosphate dehydrogenase-specific cDNA by polymerase chain reaction (PCR) amplification using the 7500 Real-Time PCR System (Applied Biosystems). The PCR primers were designed according to the protocol described by Sigma-Aldrich Japan (Tokyo, Japan) (**Table 1**).

Production of Influenza Virus Pneumonia

Influenza virus A/Puerto Rico/8/34 (H1N1) was used throughout the experiments. The virus was propagated in 10-day-old embryonated chicken eggs. The virus titer was quantitated by plaque assay using Madin–Darby canine kidney cells. Nine-week-old mice were anesthetized by intraperitoneal injection of ketamine and pentobarbital, as described previously. Then they were inoculated intranasally with H1N1 suspended in 25-μL sterile phosphate-buffered saline (PBS). The doses of influenza virus were 300 plaque-forming units for survival rate analysis and 1000 plaque-forming units for lung lavage and pathological analyses. The animals were allowed to recover thereafter. The day of virus inoculation was defined as day 0.

Administration of rhTRX-1

Recombinant human TRX-1 was supplied by Redox Bio Science, Ltd. (Kyoto, Japan). The quality and purity of the rhTRX-1 used for the present experiments were approved by Pharmaceuticals and Medical Devices Agency in Japan, which is equivalent to the Food and Drug Administration in the United States. Its safety and kinetics were established in preclinical studies using animal models (15).

TABLE 1. Selected Probes and Primers for TaqMan Amplification

Murine thioredoxin-1	TaqMan probe	5′-GCGTGTGGCTCCCTCCCCGCAAC-3′
	Forward primer	5′-GATCCATTTCCATCTGGTTCTGC-3′
	Reverse primer	5′-CAGAGAAGTCCACCACGACAAG-3′
Murine glyceraldehyde-3-phosphate dehydrogenase	TaqMan probe	5′-6ATGCCCCCATGTTTGTGATGGGTGT-3′
	Forward primer	5′-TCACCACCATGGAGAAGGC-3′
	Reverse primer	5′-GCTAAGCAGTTGGTGGTGCA-3′
Influenza virus type A (M gene)	TaqMan probe	5′-6CCCTCAAAGCCGAGATCGCACAGAGAC-3′
	Forward primer	5′-CGTTCTCTCTATCATCCCGTCAG-3′
	Reverse primer	5′-GGTCTTGTCTTTAGCCATTCCATG-3′

In preliminary experiments, 9-wk-old mice were administered rhTRX-1 intraperitoneally (40 μg in 100-μL PBS) and at 0 hr (untreated), 1, 3, or 6 hrs after administration (three to six mice at each time point) they were anesthetized and blood and the left lung were sampled for measurement of rhTRX-1 using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Redox Bio Science, Ltd.) (15, 19). The ELISA did not crossreact with murine TRX-1. The lung tissues were homogenized in 1 mL of lysis buffer. The concentrations in the sera and the lung homogenate supernatants at 0, 1, 3, and 6 hrs after the rhTRX-1 administration were, respectively, <0.5, 1237 ± 298, 97 ± 23, and 18 ± 3 ng/mL (sera) and <0.5, 62 ± 21, 20 ± 10, and 20 ± 9 ng/mL (lung tissues). In accordance with our previous work (19), a bolus intraperitoneal injection of 40 μg of rhTRX-1 led to systemic delivery and lung deposition in mice.

Murine TRX-1 mRNA Expression in the Lung After rhTRX-1 Administration

A group of 9-wk-old mice was administered rhTRX-1 intraperitoneally (40 μg in 100-μL PBS). At 0 hr (untreated), 1, 3, or 6 hrs after rhTRX-1 administration, the mice were anesthetized and the left lung was sampled for analysis of murine TRX-1 and glyceraldehyde-3-phosphate dehydrogenase mRNA expression as described previously (three to five mice at each time point).

Treatment With rhTRX-1 for H1N1-Inoculated Mice

The C57BL/6 mice were divided randomly into a vehicle-treated group (control group) and an rhTRX-1-treated group (treatment group).

Survival Rate Analysis

The vehicle (100-μL PBS) or rhTRX-1 (40 μg in 100-μL PBS) was administered intraperitoneally every second day from day −1 (the day before H1N1 inoculation) to day 13 (ten mice per group). Survival was observed until day 14. No other parameter was measured in these mice.

Lung Lavage and Pathological Analyses

The vehicle (100-μL PBS) or rhTRX-1 (40 μg in 100-μL PBS) was administered intraperitoneally on days −1, 1, and 3. Lung lavage and pathological analyses were performed on days 1 (24 hrs), 3 (72 hrs), and 5 (120 hrs) after H1N1 inoculation (five mice per group at each time point).

Lung Lavage Analysis

The mice were anesthetized by intraperitoneal injection of ketamine and pentobarbital as previously described. Blood was sampled for measurement of hydroperoxides (described subsequently). Then the left lung hilus was ligated and the right lung was lavaged twice with 500 μL cold Hanks' balanced salt solution through a 20-gauge cannula. The recovered lavage was collected and centrifuged at 2000 rpm for 10 mins at 4°C, and the supernatant was stored at −80°C for measurement of cytokines (described subsequently). The total cell number in the lavage fluid was calculated from the cell number in the 200-μL sediment. Cell differentiation was examined using Diff-Quick staining (Dade Behring, Newark, NJ) for at least 200 cells on a smear prepared from the sediment. The percentage of neutrophils was determined. The total neutrophil number in the lavage fluid was calculated and expressed per animal.

Lung Pathological Analysis

The upper portion of the left lung was fixed in buffered 4% paraformaldehyde solution and then embedded in paraffin. Sections (4-μm slices) including a cut at the hilus were stained with hematoxylin and eosin for light microscopy. The histological examination procedure was similar to that described by Xu et al (20). Four readily identifiable pathological processes were graded semiquantitatively on a scale of 0 to 4: alveolar and interstitial edema, hemorrhage, margination and infiltration of inflammatory cells, and formation of bronchiolitis; a score of 0 represented normal lung; 1 represented mild; 2 was moderate; 3 was severe; and 4 denoted very severe changes. For each mouse, the lung injury score was calculated by adding the individual grades (the mean value for four sections) for each category. The histology was reviewed by two of the authors (M. Yashiro, A.M.) in a blinded manner. The middle portion of the left lung was excised and soaked in RNAlater. Total RNA was extracted, and 1 μg of total RNA was reverse-transcribed to cDNA according to the procedure previously described. The samples were analyzed for virus copies using the 7500 Real-

Time PCR System. The PCR primers were designed according to the protocol of Sigma-Aldrich Japan (Table 1).

Immunohistochemical Study

Immunohistochemical analysis was performed using the other upper lung sections (4-μm slices) that were obtained from vehicle-treated and rhTRX-1–treated mice on day 3 (72 hrs) after H1N1 inoculation. The lung sections obtained from 9-wk-old nonvirus-inoculated mice served as controls. Antibodies against granulocyte-differentiation antigen (Gr-1) (BioLegend, San Diego, CA) (21) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Japan Institute for the Control of Aging, Shizuoka, Japan) (22) were used, respectively, for detection of neutrophil infiltration and cellular DNA oxidation according to the manufacturers' instructions. The results were evaluated by two of the authors (M. Yashiro, A.M.) in a blinded manner.

Measurement of Cytokines

Tumor necrosis factor (TNF)-α, a proinflammatory cytokine, participates in important processes involved in the inflammatory response. chemokine (C-X-C motif) ligand 1 (CXCL1), a member of the chemokine (C-X-C motif) (CXC) chemotactic cytokine family, plays a pivotal role in the activation and extravasation of neutrophils (23). Concentrations of TNF-α and CXCL1 were measured using sandwich ELISA methods in lung lavage fluids obtained from vehicle-treated and rhTRX-1-treated mice on days 1 (24 hrs), 3 (72 hrs), and 5 (120 hrs) after H1N1 inoculation (five mice per group at each time point). Those concentrations were also measured in the right lung homogenate supernatants obtained from the same mice on day 3 (72 hrs) (five mice per group). The ELISA procedure was referred to that described by Matsukawa et al (23). The captured antibodies, detection antibodies, and recombinant cytokines were purchased from R&D Systems (Minneapolis, MN). The ELISAs used for this study did not crossreact with other available murine cytokines. They consistently detected cytokine concentrations higher than 10 pg/mL.

Measurement of Serum Hydroperoxides

Blood was sampled from vehicle-treated and TRX-1–treated mice on days 1 (24 hrs), 3 (72 hrs), and 5 (120 hrs) after H1N1 inoculation (five mice per group at each time point). Blood was also sampled from another group of 9-wk-old nonvirus-inoculated mice given vehicle (100-μL PBS) intraperitoneally 24 hrs before (ten mice). Serum was prepared. The serum concentration of hydroperoxides (whole oxidant capacity of serum against N,N-diethylparaphenylene-diamine in acidic buffer) was measured using the Free Radical Analytical System (Diacron International, Grosseto, Italy) (24). The measurement unit was CARR U. It has been established that 1 CARR U corresponds to 0.08 mg/dL hydrogen peroxide (25).

Cell Biological Study

MLE-12 cells (ATCC, Manassas, VA), a SV40-transformed murine lung epithelial cell line, were grown to confluence in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Gibco-BRL, Grand Island, NY). The MLE-12 cells were not inoculated or were inoculated with H1N1 (at multiplicity of infection of 10) for 6 hrs. The H1N1-inoculated cells were not treated or were treated with rhTRX-1 (at doses of 10 or 100 ng/mL) simultaneously. Expression of TNF-α and CXCL1 mRNA was analyzed using reverse transcription and real-time quantitative PCR (ABI 7700 Sequence Detector System; Applied Biosystems), as described previously by Ito et al (26).

Treatment With rhTRX-1 After H1N1 Inoculation (Therapeutic Protocols)

Other groups of 9-wk-old mice were used in the therapeutic protocols. The dose of influenza virus was 300 plaque-forming units. The intraperitoneal administration of vehicle (100-μL PBS) or rhTRX-1 (40 μg in 100-μL PBS) was started 30 mins (14 mice per group) or 4 hrs (11 mice per group) after H1N1 inoculation (day 0) and repeated every second day until day 12. Survival was observed until day 14.

Statistical Analysis

All data are expressed as mean ± SEM and compared using unpaired *t* test or one-way analysis of variance or two-way analysis of variance followed by Bonferroni's posttest where appropriate. Survival curves were analyzed using the Kaplan–Meier log-rank test. Differences for which $p < 0.05$ were considered significant. All statistical calculations were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

RESULTS

Regional Distribution of mRNA Expression of TRX-1

Figure 1*A* shows the messenger RNA (mRNA) expression of TRX-1 in normal mice for various organs. The level of TRX-1 expression in the lung was the highest: several times higher than that in the other organs including the brain, liver, kidney, and spleen. Results suggest that TRX-1 plays some physiologically important regulatory role in the animal lung.

Murine TRX-1 mRNA Expression in the Lung After rhTRX-1 Administration

Figure 1*B* presents changes of mRNA expression of TRX-1 in the lung of normal mice after rhTRX-1 administration. The intrinsic mRNA expression of TRX-1 was suppressed in the lung after a bolus intraperitoneal injection of rhTRX-1 (40 μg). The suppressive effect was statistically significant at 1 hr and 3 hrs after the administration compared with the pretreatment level.

Effects of rhTRX-1 on Survival Rate and Viral Load in the Lung After H1N1 Inoculation

Comparison of survival curves using the Kaplan–Meier log-rank test showed a significant difference between the vehicle-treated group and the rhTRX-1-treated group (**Fig. 2*A***). The rhTRX-1 treatment significantly improved the survival rate of H1N1-inoculated mice. All ten control mice died from day 7 to day 9, but four (40%) of ten rhTRX-1-treated mice survived over 14 days after H1N1 inoculation. Of note, all animals in both groups survived over 5 days (120 hrs) after inoculation. All the mice that survived until day 14 recovered health thereafter.

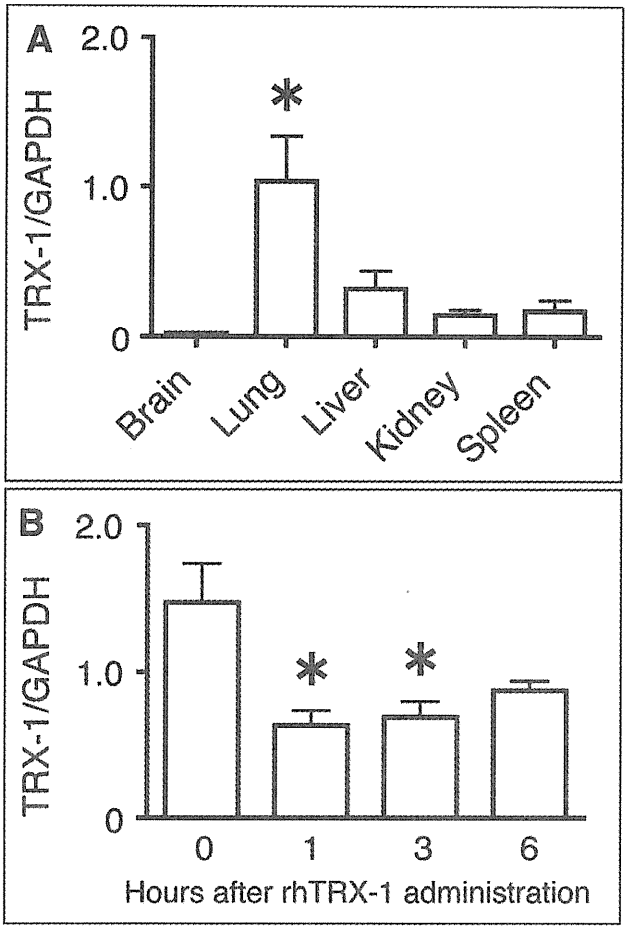

Figure 1. Thioredoxin (TRX)-1 messenger RNA (mRNA) expression in mice. **A**, Regional distribution of TRX-1 mRNA expression. Data are expressed as a percentage relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and represent the mean (SE) of five to ten independent experiments. $^*p < 0.05$ vs. brain, kidney, and spleen by one-way analysis of variance (ANOVA) with Bonferroni's posttest. **B**, Changes of TRX-1 mRNA expression in the lung after rhTRX-1 administration. Data are expressed as a percentage relative to GAPDH mRNA and represent the mean (SE) of three to five independent experiments. $^*p < 0.05$ vs. 0 hr by one-way ANOVA with Bonferroni's posttest.

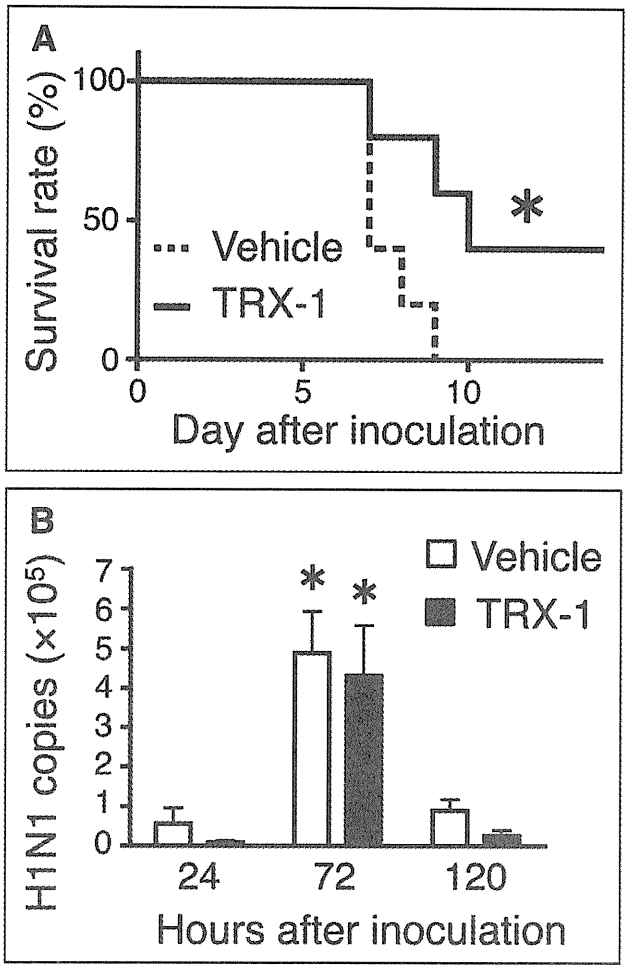

Figure 2. Effects of recombinant human thioredoxin (TRX)-1 treatment on survival rate and viral load in the lung after H1N1 inoculation. **A**, Survival rate. All mice in the vehicle group (ten mice, *broken line*) died from day 7 to day 9. In the TRX-1 group (ten mice, *solid line*), four (40%) survived until day 14. $^*p < 0.05$ vs. vehicle. **B**, Viral load in the lung. Data represent the mean (SE) of five independent experiments. $^*p < 0.01$ vs. 24 hrs, 120 hrs by one-way analysis of variance (ANOVA) with Bonferroni's posttest. Two-way ANOVA was significant for time, $p < 0.001$, but not for treatment or time × treatment. No significant difference was detected between the two groups at any time point by Bonferroni's posttest.

The viral load in the lung increased significantly from 24 to 72 hrs after inoculation, decreasing thereafter in control mice (Fig. 2*B*). A similar tendency was observed in rhTRX-1-treated mice. The viral load in rhTRX-1-treated mice was almost comparable to that in control mice at each time point. These results indicate that rhTRX-1 treatment improves the survival rate of H1N1-inoculated mice significantly, although the treatment does not affect propagation of the influenza virus in the lungs of these animals.

Effects of rhTRX-1 on Lung Histology After H1N1 Inoculation

H1N1-inoculated control mice presented diffuse edema and inflammatory cellular infiltration in alveoli and interstitium of the lung, hemorrhage, and thickened airways at 72 hrs after inoculation. The rhTRX-1 treatment attenuated the histological changes in the lung (**Fig. 3***A*). The lung injury score increased significantly from 24 to 72 hrs and 120 hrs in the control group ($p < 0.01$ at 24 hrs vs. 72, 120 hrs, one-way analysis of variance with Bonferroni's posttest) (Fig. 3*B*). In the treatment group, the score increased significantly from 24 to 72 hrs ($p < 0.05$), but the score at 120 hrs was not significantly different from that at 24 hrs. The lung injury score remained significantly lower in rhTRX-1-treated mice than in control mice. These histological analyses indicate that rhTRX-1 treatment significantly attenuates the degree of acute lung injury in H1N1-inoculated mice.

Effects of rhTRX-1 on Neutrophil Infiltration in the Lung After H1N1 Inoculation

The neutrophil number in lung lavage fluid increased significantly from 24 to 72 hrs after virus inoculation in control group ($p < 0.01$, one-way analysis of variance with Bonferroni's posttest). The neutrophil number at 120 hrs was not significantly

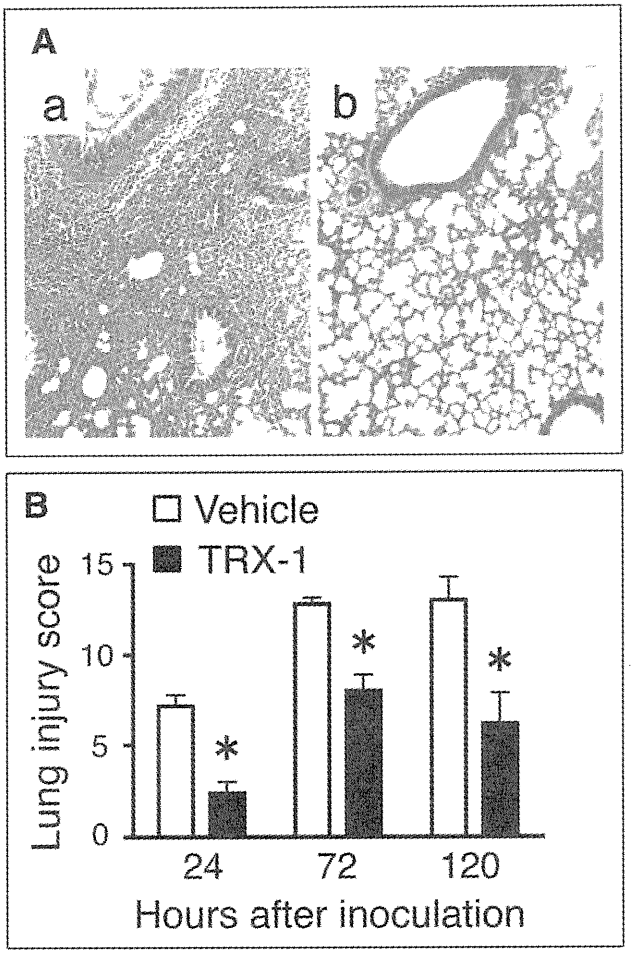

Figure 3. Effects of recombinant human thioredoxin (TRX)-1 treatment on lung histology after H1N1 inoculation. **A**, Photomicrographs of lung tissue samples stained with hematoxylin and eosin at 72 hrs after H1N1 inoculation. They are representative of five independent experiments. (*a*) Vehicle-group lung tissue showed diffuse alveolar and interstitial edema, inflammatory cellular infiltration, hemorrhage, and bronchiolitis. (*b*) In TRX-1 group lung tissue, these features were much less severe. Original magnification, ×200. **B**, Lung injury scores. Lung histological changes were graded from 0 to 4 in four categories. Lung injury scores were calculated by adding the individual grades for each category. Data represent the mean (SE) of five independent experiments. Two-way analysis of variance was significant for time, $p < 0.001$; treatment, $p < 0.001$; but not for time × treatment. $^*p < 0.01$ vs. vehicle by Bonferroni's posttest.

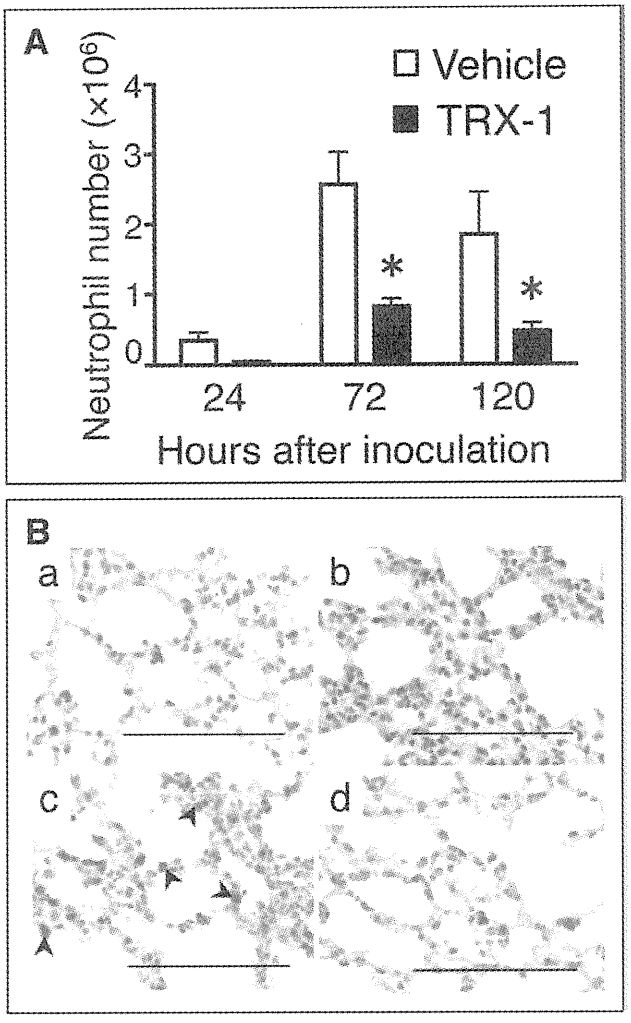

Figure 4. Effects of recombinant human thioredoxin (TRX)-1 treatment on neutrophil infiltration in the lung after H1N1 inoculation. **A**, Neutrophil numbers in lung lavage fluid. TRX-1 treatment significantly decreased the neutrophil number in lung lavage fluid at 24 hrs and 72 hrs after H1N1 inoculation. Data represent the mean (SE) of five independent experiments. Two-way analysis of variance was significant for time, $p < 0.005$; treatment, $p < 0.001$; but not for time × treatment. $^*p < 0.05$ vs. vehicle by Bonferroni's posttest. **B**, Photomicrographs of lung tissue samples stained with granulocyte-differentiation antigen (Gr-1) at 72 hrs after H1N1 inoculation. They are representative of three independent experiments: (*a*) no H1N1 inoculation; (*b*) H1N1 inoculation, no primary antibody; (*c*) H1N1 inoculation, vehicle group; and (*d*) H1N1 inoculation, TRX-1 group. *Arrowheads* indicate positively stained (*brown*) cells. Scale bars = 100 μm.

different from that at 72 hrs (**Fig. 4*A***). In the treatment group, the neutrophil number increased significantly from 24 to 72 hrs and decreased significantly thereafter ($p < 0.01$ at 72 hrs vs. 24, 120 hrs). The neutrophil number remained lowered in rhTRX-1-treated mice compared with control mice. The difference reached statistical significance at 72 and 120 hrs after inoculation. Histologically, influenza virus inoculation increased neutrophil infiltration in the lung at 72 hrs after inoculation. The rhTRX-1 treatment almost reversed this effect (Fig. 4*B*). These results indicate that rhTRX-1 treatment significantly attenuates the neutrophil infiltration in the lung of H1N1-inoculated mice.

Effects of rhTRX-1 on Cytokine Production in the Lung After H1N1 Inoculation

The TNF-α and CXCL1 concentrations in lung lavage fluid were significantly lower in rhTRX-1-treated mice than in control mice at 72 hrs after inoculation (**Fig. 5*A–B***). In lung tissue analyses, both cytokine concentrations were lowered significantly by rhTRX-1 treatment at 72 hrs after inoculation (Fig. 5*C–D*). These results indicate that rhTRX-1 treatment significantly attenuates the inflammatory cytokine production in the lung of H1N1-inoculated mice.

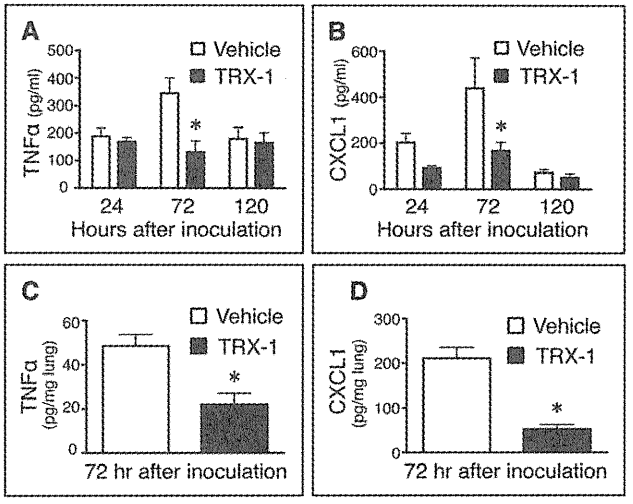

Figure 5. Effects of recombinant human thioredoxin (TRX)-1 treatment on cytokine production in the lung after H1N1 inoculation. **A**, Tumor necrosis factor (TNF)-α concentrations in lung lavage fluid. Data represent the mean (SE) of five independent experiments. Two-way analysis of variance (ANOVA) was significant for treatment, $p < 0.05$; time × treatment, $p < 0.05$; but not for time. *$p < 0.01$ vs. vehicle by Bonferroni's posttest. **B**, Chemokine (C-X-C motif) ligand 1 concentrations in lung lavage fluid. Data represent the mean (SE) of five independent experiments. Two-way ANOVA was significant for time, $p < 0.001$; treatment, $p < 0.05$; but not for time × treatment. *$p < 0.01$ vs. vehicle by Bonferroni's posttest. **C** and **D**, Cytokine concentrations in lung tissue samples. Data represent the mean (SE) of five independent experiments. *$p < 0.01$ vs. vehicle by unpaired *t* test.

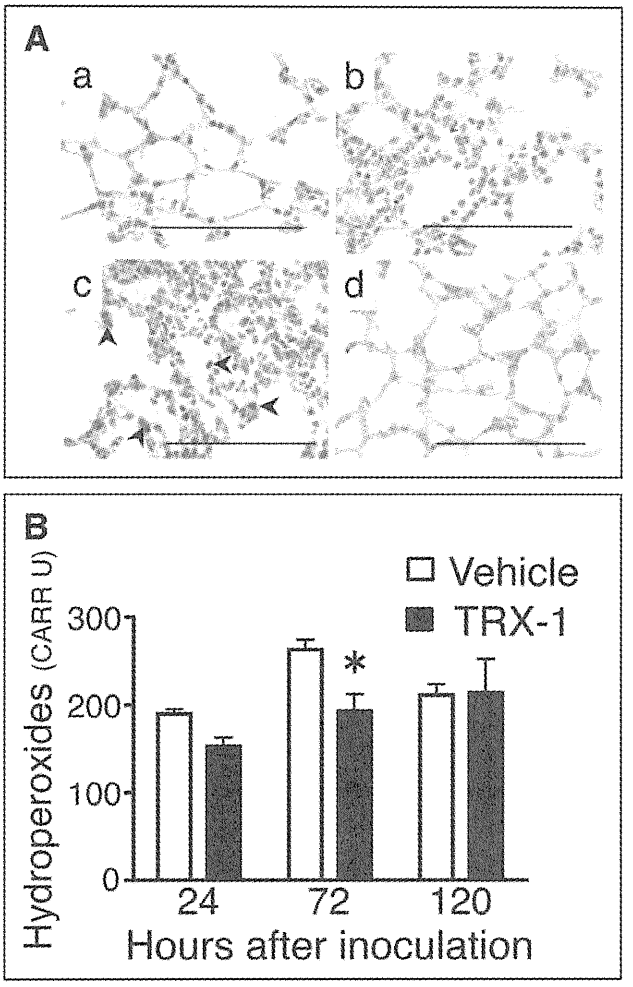

Figure 6. Effects of recombinant human thioredoxin (TRX)-1 treatment on oxidative stress markers after H1N1 inoculation. **A**, Photomicrographs of lung tissue samples stained with 8-hydroxy-2′-deoxyguanosine at 72 hrs after H1N1 inoculation. They are representative of three independent experiments: (*a*) no H1N1 inoculation; (*b*) H1N1 inoculation, no primary antibody; (c) H1N1 inoculation, vehicle group; and (*d*) H1N1 inoculation, TRX-1 group. *Arrowheads* indicate positively stained (brown) cells. Scale bars, 100 μm. **B**, Hydroperoxides in blood serum. Data represent the mean (SE) of five independent experiments. Two-way analysis of variance was significant for time, $p < 0.05$, but not for treatment or time × treatment. *$p < 0.05$ vs. vehicle by Bonferroni's posttest.

Effects of rhTRX-1 on Oxidative Stress Markers After H1N1 Inoculation

Histologically, H1N1 inoculation increased 8-OHdG formation in the lung at 72 hrs after inoculation. The 8-OHdG formation was observed primarily in infiltrating cells and occasionally in lung epithelial cells. The rhTRX-1 treatment almost reversed this effect (**Fig. 6*A***). Serum concentration of hydroperoxides was significantly lower in rhTRX-1-treated mice than in control mice at 72 hrs after inoculation (Fig. 6*B*). Concentrations at 24, 72, and 120 hrs after inoculation in either group were significantly higher than that (122 ± 6 CARR U) in nonvirus-inoculated mice ($p < 0.05$ in each, unpaired *t* test). These results indicate that rhTRX-1 treatment significantly attenuates the oxidative stress enhancement that is observed in H1N1-inoculated mice.

Effects of rhTRX-1 on Cytokine mRNA Expression in H1N1-Inoculated MLE-12 Cells

Expression of cytokine mRNA was analyzed by reverse-transcription PCR in MLE-12 cells. The TNF-α and CXCL1 mRNA accumulated significantly at 6 hrs after inoculation (**Fig. 7*A–B***). Simultaneous rhTRX-1 treatment (at doses of 10, 100 ng/mL) almost abolished this effect. These results indicate that rhTRX-1 treatment significantly attenuates the increase in cytokine mRNA expression in H1N1-inoculated murine lung epithelial cells.

Treatment With rhTRX-1 After H1N1 Inoculation (Therapeutic Protocols)

The rhTRX-1 treatment started 30 mins after virus inoculation significantly improved the survival rate of H1N1-inoculated mice ($p < 0.05$ vs. control) (**Fig. 8*A***). Only one of 14 control mice (7%) survived over 14 days after H1N1 inoculation, but six (43%) of 14 rhTRX-1-treated mice survived. However, the rhTRX-1 treatment started 4 hrs after inoculation had no effect on the survival rate. None of 11 control mice and only two (18%) of 11 rhTRX-1-treated mice survived >14 days after H1N1 inoculation (Fig. 8*B*).

DISCUSSION

TRX-1 is a ubiquitously expressed, multifunctional protein that has a redox-active dithiol–disulfide within the conserved -Cys-Gly-Pro-Cys- sequence. TRX-1 protects cells against oxidative stress by scavenging ROS in concert with peroxiredoxins and prevents cellular apoptosis by inhibiting apoptosis signal-regu-

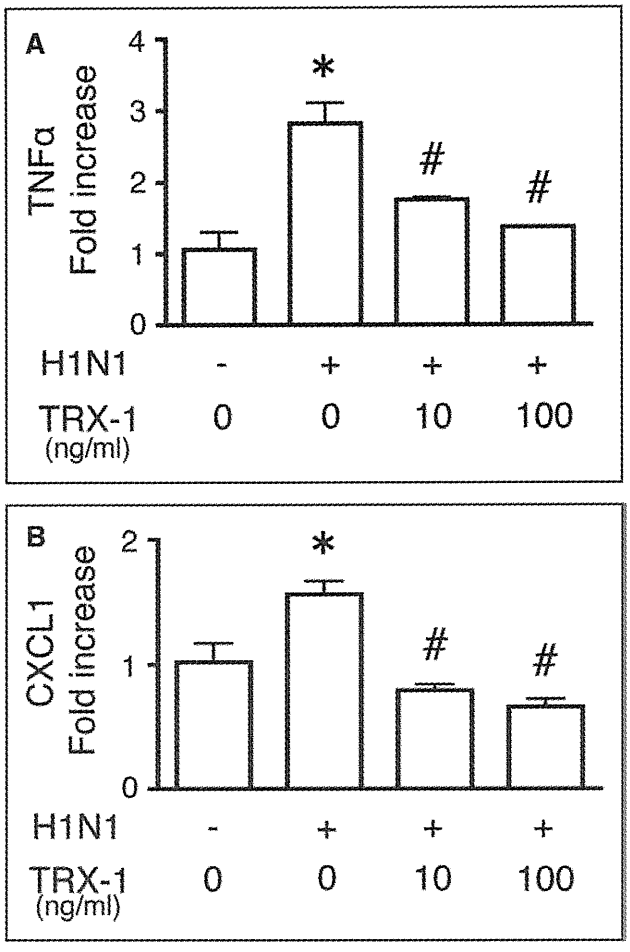

Figure 7. A and **B**, Effects of recombinant human thioredoxin (TRX)-1 treatment on cytokine mRNA expression in H1N1-inoculated lung epithelial cells (MLE-12). Cells were inoculated with H1N1 (at multiplicity of infection of 10) for 6 hrs. They were not treated or were treated with TRX-1 simultaneously. Data are expressed as a percentage relative to the basal expression level. $n = 4$ per group. $^{*}p < 0.01$ vs. no H1N1 inoculation (medium alone) by unpaired t test; $\#p < .001$ vs. H1N1 inoculation, no TRX-1 treatment by one-way analysis of variance with Bonferroni's posttest. TNF = tumor necrosis factor; CXCL1 = chemokine (C-X-C motif) ligand 1.

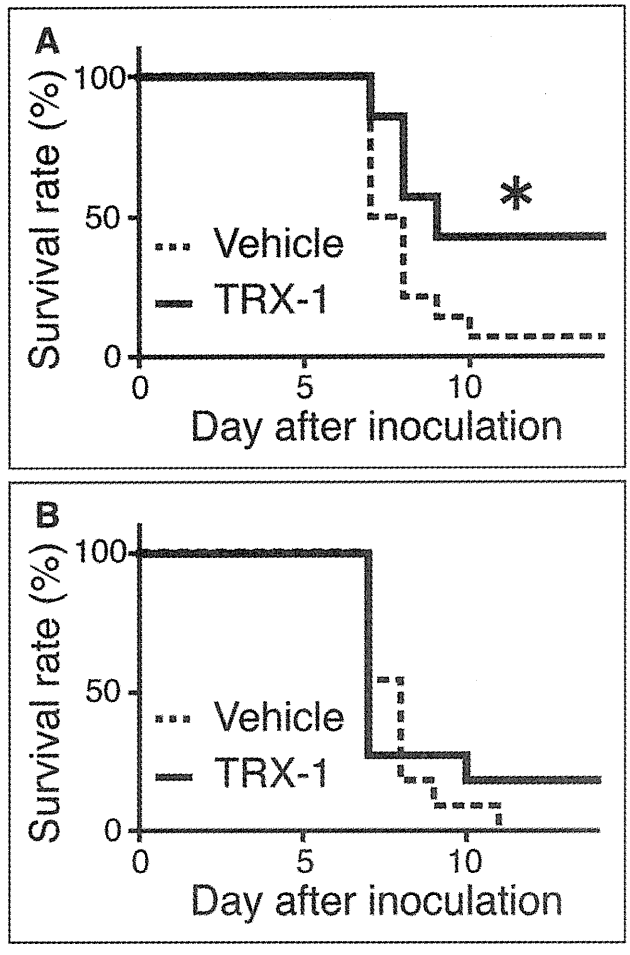

Figure 8. Therapeutic effects of recombinant human thioredoxin (TRX)-1 treatment started 30 mins or 4 hrs after H1N1 inoculation on mouse survival rate. **A**, TRX-1 treatment started 30 mins after virus inoculation significantly improved the survival rate of H1N1-inoculated mice (14 mice per group). $^{*}p < 0.05$ vs. vehicle. **B**, The TRX-1 treatment started 4 hrs after inoculation had no effect on the survival rate (11 mice per group).

lating kinase 1 (15). Furthermore, TRX-1 suppresses inflammation by regulating neutrophil activation and extravasation and exerts the anti-inflammatory effect (15, 16). In the clinical field, extracellular concentrations of TRX-1 have been measured in various conditions characterized by oxidative stress and inflammation, including sepsis, viral infection, autoimmune disease, ischemia–reperfusion injury, and acute lung injury (27–30). These studies document that the TRX-1 concentrations are elevated in patients with these diseases and that they are correlated significantly with the activity of such diseases.

Overexpression of human TRX-1 in transgenic mice induces resistance to harmful conditions, including ischemic brain damage, Adriamycin-induced cardiotoxicity, ischemia–reperfusion renal injury, and cerulein-induced pancreatitis (31–34). More importantly, human TRX-1 transgenic mice are more resistant than control mice to proinflammatory cytokine-, bleomycin-, diesel exhaust particle-, or cigarette smoke-induced lung injury (19, 35, 36) and to influenza virus-induced pneumonia (18). Administration of rhTRX-1 is also effective in animal models, especially for acute lung injury, including proinflammatory cytokine-, bleomycin-, or cigarette smoke-induced inflammatory injury, ovalbumin-induced airway hyperresponsiveness and inflammation, and lipopolysaccharide-induced bronchoalveolar neutrophil infiltration (19, 36–38). All these results demonstrate that TRX-1 has potent protective effects on oxidative stress-associated or inflammation-associated lung disorders in animals.

Influenza virus infections are responsible for numerous pneumonia cases every year. In severe cases, they can cause death (1–3). Influenza virus causes death of infected cells by cytopathology. Furthermore, when the immune system responds to the infection exuberantly, additional lung damage and overwhelming systemic illness are likely to ensue. A marked increase in concern has occurred in relation to the possibility of future severe pandemics. Therefore, it is crucial to identify new therapeutic strategies for influenza (4).

This study demonstrated, for the first time, the protective effects of rhTRX-1 administration in a murine model of acute lung injury induced by influenza virus infection. Intranasal instillation of influenza A virus (H1N1) was used to produce viral pneumonia in this model. Every second day, 40 μg of rhTRX-1 was administered intraperitoneally. At the beginning of the study, we examined the regional distribution of mRNA expression of TRX-1 in normal mice. We found that TRX-1 expression was at the highest level in the lung. Results suggest that the endogenous TRX-1 system plays some physiologically important regulatory role in the lung and that lung disease might be a target for this protein (15). Results also show that the TRX-1 expression in the lung was suppressed significantly after intraperitoneal injection of rhTRX-1. Administration of rhTRX-1 producing elevated blood concentrations (mean: 1237 ng/mL), which are approximately ten times higher than those in oxidative and inflammatory disorders in humans (40–140 ng/mL) (15), is likely to suppress the endogenous TRX-1 system in the animal lung.

Intraperitoneal administration of rhTRX-1 significantly improved the survival rate of H1N1-inoculated mice. The viral load in the lung increased from 24 to 72 hrs after inoculation, confirming the occurrence of influenza pneumonia in both vehicle-treated (control) and rhTRX-1-treated mice. Notably, the viral load in rhTRX-1-treated mice was almost comparable to that of control mice at each time point of the study. The rhTRX-1 treatment did not affect propagation of influenza virus in the animal lung. The result is consistent with previous reports in which administration of N-monomethyl-L-arginine, overexpression of extracellular superoxide dismutase, or adenovirus-mediated transfer of heme oxygenase-1 was used as a therapeutic tool against influenza pneumonia in mice (10–12). The occurrence of influenza pneumonia was documented histologically in mice subjected to H1N1 inoculation in this study. The H1N1-inoculated control mice presented characteristic changes of acute lung injury: diffuse edema and inflammatory cellular infiltration in alveoli and interstitium of the lung, hemorrhage, and thickened airways (20). Particularly, the lung injury score remained at high levels at 24, 72, and 120 hrs after inoculation. The rhTRX-1 treatment attenuated the histological changes and the lung injury score, confirming histologically that rhTRX-1 ameliorates H1N1-induced pneumonia in mice.

Previous studies revealed that influenza virus infection caused a marked increase in the cell number in lung lavage fluid and demonstrated that this increase primarily reflected an increase in neutrophils and macrophages (9–12). In this study, we measured the neutrophil number in lung lavage fluid obtained from the animals. In control mice, the neutrophil number increased from 24 to 72 hrs and remained high at 120 hrs. The rhTRX-1 treatment suppressed the increase in neutrophils. Histological examination revealed much less neutrophil infiltration to lung parenchyma in rhTRX-1-treated mice. Accumulation of activated neutrophils in the lung is an early critical step in the inflammatory process of acute lung injury. According to our previous studies (15, 16, 34, 36, 38), elevated levels of circulating TRX-1 effectively suppress neutrophil extravasation into sites of inflammation. The protective effects of rhTRX-1 on H1N1-induced pneumonia are partly attributed to its antichemotactic action for neutrophils.

Increased synthesis and secretion of inflammatory mediators contribute to the overall pathology of lung injury. Several cytokines apparently play crucial roles in the acute and uncontrollable inflammatory process of influenza pneumonia (1, 2, 4, 39, 40). In this study, we measured TNF-α (a proinflammatory cytokine) and CXCL1 (a CXC chemotactic cytokine) concentrations in lung lavage fluid at 24, 72, and 120 hrs after inoculation and lung homogenate supernatants at 72 hrs. The rhTRX-1 treatment suppressed the concentrations of these cytokines. Additionally, H1N1 inoculation increased the TNF-α and CXCL1 mRNA expression in MLE-12 cells (a SV40-transformed murine lung epithelial cell line) directly; simultaneous rhTRX-1 treatment almost abolished this effect. The in vivo and in vitro experimentally obtained results indicate that rhTRX-1 treatment attenuates the increase in cytokine expression and secretion in the lung of H1N1-inoculated mice.

Evaluating oxidative stress status in the experimental animals was crucial because the redox imbalance was regarded as a key lung injury pathway in influenza pneumonia (4, 8–14, 18). The DNA base-modified product 8-OHdG is a marker for oxidative stress in tissue samples (6, 22, 36). In the present study, 8-OHdG formation was intense in the lung of H1N1-inoculated control mice; 8-OHdG was detected in infiltrating inflammatory cells and lung epithelial cells. Serum concentration of hydroperoxides is a marker for oxidative stress in the whole body (24, 25). A significant increase in serum hydroperoxides occurred in H1N1-inoculated mice, implying the presence of systemic oxidative stress enhancement. The rhTRX-1 treatment suppressed 8-OHdG formation in the lung and serum concentration of hydroperoxides in H1N1-inoculated mice. These results indicate that oxidative stress enhancement is indeed involved in the inflammatory process of H1N1-induced pneumonia and that the protective effects of rhTRX-1 are partly attributed to its potent antioxidative action.

In our murine model, influenza pneumonia is characterized histologically by intense infiltration of inflammatory cells (mainly neutrophils). Activated neutrophils, macrophages, and respiratory tract epithelial cells might release excess amounts of bioactive substances, including cytokines/chemokines, ROS, and tissue degradative enzymes, and induce acute lung inflammatory disease (4, 5). Presumably, oxidative injury by the inflamed cells is targeted to the vascular endothelium, leading to lung edema and hemorrhage. Based on these considerations, it is plausible that rhTRX-1 administration ameliorates the lethal effects of influenza A virus (H1N1)-induced pneumonia in mice through antioxidative and anti-inflammatory actions, including the antichemotactic effect for neutrophils.

For potential clinical use, we also examined whether treatment with rhTRX-1 after H1N1 inoculation is efficacious for protection. As a therapeutic protocol, vehicle or rhTRX-1 (40 μg) was given intraperitoneally to mice every second day from 30 mins after inoculation (day 0) to day 12. Administration of rhTRX-1 started 30 mins after H1N1 inoculation exerted a

significant survival-promoting effect. Therefore, it is conceivable that rhTRX-1 provided 30 mins after virus inoculation ameliorated H1N1-induced lung inflammatory injury in mice. However, administration of rhTRX-1 started 4 hrs after virus inoculation had no such therapeutic effect.

The beneficial effects on H1N1-inoculated mice observed in this study might not be specific to rhTRX-1, but to the other antioxidative agents. The effects of a well-known glutathione precursor, N-acetylcysteine (41), on H1N1-induced pneumonia have been evaluated. The vehicle (100 μL PBS) or N-acetylcysteine (200 mg/kg in 100 μL PBS) (42) was administered intraperitoneally to another group of 9-wk-old mice every second day from day −1 to day 13. With this intermediate dose, N-acetylcysteine did not alter the survival rate or lung injury score (at 72 hrs) of H1N1-inoculated mice (data not shown). Results of previous studies have indicated that N-acetylcysteine has protective effects in the murine model of influenza pneumonia, especially when administered in combination with antiviral drugs (4, 43, 44). N-acetylcysteine reportedly has beneficial effects in various clinical conditions characterized by glutathione deficiency or oxidative stress enhancement (41). It is therefore highly possible that higher doses of N-acetylcysteine can efficiently reduce the lethal effects of influenza virus infection in our experimental model. This issue deserves further examination.

Our results raise the possibility that loss of TRX-1 function aggravates influenza virus-induced pneumonia, although complete TRX-1 deficiency results in early embryonic lethality (45). The cecal ligation and puncture septic murine model revealed that neutralization of endogenous TRX-1 impaired mice survival but that treatment with rhTRX-1 enhanced mice survival (30). Although extracellular TRX-1 concentrations were elevated in many oxidative and inflammatory disorders (27–30), plasma TRX-1 concentrations were persistently low in children with meningococcal septic shock, possibly because of a genetic predisposition (46). Plasma TRX-1 concentrations were lower in patients with neutropenia/sepsis than in patients with systemic inflammatory response syndrome/sepsis (47). Although speculative, the low TRX-1 condition might contribute to the devastating nature of sepsis. At present, no information related to the concentration of TRX-1 in blood or lung lavage fluid from patients with influenza pneumonia is available.

We reported that endogenous TRX-1 expression was induced by natural substances including estrogen, prostaglandins and cyclic AMP, geranylgeranylacetone (an antiulcer drug derived from a natural plant constituent), and temocapril (a nonsulfhydryl-containing angiotensin-converting enzyme inhibitor) (48). We reported also that the cytoprotective action of TRX-1 was augmented after S-nitrosation (49). Furthermore, concentrations of TRX-1 (mean: 268 ng/mL) and nitrite/nitrate (mean: 479 μmol/L) in early human milk were found to be approximately ten times higher than those (mean: 20 ng/mL, 40 μmol/L, respectively) in blood of healthy adults (50, 51)

The protective effects of such "TRX-1 inducers" or "TRX-1 donors" against infectious disorders, including influenza pneumonia, warrant further study.

The present results suggest that systemic administration of rhTRX-1 can be clinically beneficial for ameliorating acute lung injury in influenza virus-infected mice. However, several concerns persist in terms of clinical relevance. First, the experimentally obtained results for animals cannot be extrapolated directly to the medical arena. Second, the survival rate of rhTRX-treated virus-inoculated mice is not entirely satisfactory, although the rhTRX-1 treatment proved to exert lung-protective effects in the mice. Last, the posttreatment experiments suggest that the therapeutic time window for rhTRX-1 might be narrow. The combination of rhTRX-1 with current anti-influenza drugs might expand the therapeutic window as well as the protective effects of rhTRX-1 against influenza infection. Further investigations are necessary to explore the future clinical application of rhTRX-1 for severe influenza pneumonia in humans.

CONCLUSIONS

Exogenous administration of redox-active protein rhTRX-1 significantly improved the survival rate and attenuated lung histological changes in the murine model of influenza pneumonia. The rhTRX-1 treatment did not affect virus propagation in the animal lung. The protective mechanism of rhTRX-1 might be explained by its potent antioxidative and anti-inflammatory actions. These results suggest that rhTRX-1 represents a possible pharmacological strategy for severe influenza virus infection in humans.

ACKNOWLEDGMENTS

We thank Dr. Tetsuya Ogino, Dr. Soichiro Fushimi, Mr. Yasuharu Arashima, Mr. Haruyuki Watanabe (Department of Pathology and Experimental Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences), Dr. Nobuaki Miyahara (Department of Allergy and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences), Dr. Keiki Ogino (Department of Public Health, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences), and Dr. Eiji Yoshihara (Department of Biological Responses, Institute for Virus Research, Kyoto University) for their excellent support for this study.

REFERENCES

1. Subbarao K: Influenza viruses. *In*: Principles and Practice of Pediatric Infectious Diseases. Third Edition. Long SS, Pickering LK, Prober CG (Eds). Philadelphia, PA, Churchill Livingstone-Elsevier, 2008, pp 1130–1138
2. Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, et al; Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Virus: Update on avian influenza A (H5N1) virus infection in humans. *N Engl J Med* 2008; 358:261–273
3. Perez-Padilla R, de la Rosa-Zamboni D, Ponce de Leon S, et al; INER Working Group on Influenza: Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. *N Engl J Med* 2009; 361:680–689
4. Uchide N, Toyoda H: Antioxidant therapy as a potential approach to severe influenza-associated complications. *Molecules* 2011; 16:2032–2052
5. Tsukahara H: Biomarkers for oxidative stress: Clinical application in pediatric medicine. *Curr Med Chem* 2007; 14:339–351

6. Toyokuni S, Akatsuka S: Pathological investigation of oxidative stress in the post-genomic era. *Pathol Int* 2007; 57:461–473
7. Beckman JS, Koppenol WH: Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am J Physiol* 1996; 271:C1424–C1437
8. Akaike T, Ando M, Oda T, et al: Dependence on O2- generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. *J Clin Invest* 1990; 85:739–745
9. Choi AM, Knobil K, Otterbein SL, et al: Oxidant stress responses in influenza virus pneumonia: Gene expression and transcription factor activation. *Am J Physiol* 1996; 271:L383–L391
10. Akaike T, Noguchi Y, Ijiri S, et al: Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci U S A* 1996; 93:2448–2453
11. Suliman HB, Ryan LK, Bishop L, et al: Prevention of influenza-induced lung injury in mice overexpressing extracellular superoxide dismutase. *Am J Physiol Lung Cell Mol Physiol* 2001; 280:L69–L78
12. Hashiba T, Suzuki M, Nagashima Y, et al: Adenovirus-mediated transfer of heme oxygenase-1 cDNA attenuates severe lung injury induced by the influenza virus in mice. *Gene Ther* 2001; 8:1499–1507
13. Shi XL, Shi ZH, Huang H, et al: Therapeutic effect of recombinant human catalase on H1N1 influenza-induced pneumonia in mice. *Inflammation* 2010; 33:166–172
14. Beck MA, Nelson HK, Shi Q, et al: Selenium deficiency increases the pathology of an influenza virus infection. *FASEB J* 2001; 15:1481–1483
15. Nakamura H, Hoshino Y, Okuyama H, et al: Thioredoxin 1 delivery as new therapeutics. *Adv Drug Deliv Rev* 2009; 61:303–309
16. Nakamura H, Herzenberg LA, Bai J, et al: Circulating thioredoxin suppresses lipopolysaccharide-induced neutrophil chemotaxis. *Proc Natl Acad Sci U S A* 2001; 98:15143–15148
17. Mitsui A, Hamuro J, Nakamura H, et al: Overexpression of human thioredoxin in transgenic mice controls oxidative stress and life span. *Antioxid Redox Signal* 2002; 4:693–696
18. Nakamura H, Tamura S, Watanabe I, et al: Enhanced resistancy of thioredoxin-transgenic mice against influenza virus-induced pneumonia. *Immunol Lett* 2002; 82:165–170
19. Hoshino T, Nakamura H, Okamoto M, et al: Redox-active protein thioredoxin prevents proinflammatory cytokine- or bleomycin-induced lung injury. *Am J Respir Crit Care Med* 2003; 168:1075–1083
20. Xu T, Qiao J, Zhao L, et al: Effect of dexamethasone on acute respiratory distress syndrome induced by the H5N1 virus in mice. *Eur Respir J* 2009; 33:852–860
21. Fleming TJ, Fleming ML, Malek TR: Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol* 1993; 151:2399–2408
22. Toyokuni S, Tanaka T, Hattori Y, et al: Quantitative immunohistochemical determination of 8-hydroxy-2′-deoxyguanosine by a monoclonal antibody N45.1: Its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab Invest* 1997; 76:365–374
23. Matsukawa A, Hogaboam CM, Lukacs NW, et al: Expression and contribution of endogenous IL-13 in an experimental model of sepsis. *J Immunol* 2000; 164:2738–2744
24. Tomofuji T, Ekuni D, Sanbe T, et al: Effects of vitamin C intake on gingival oxidative stress in rat periodontitis. *Free Radic Biol Med* 2009; 46:163–168
25. Pasquini A, Luchetti E, Marchetti V, et al: Analytical performances of d-ROMs test and BAP test in canine plasma. Definition of the normal range in healthy Labrador dogs. *Vet Res Commun* 2008; 32:137–143
26. Ito T, Schaller M, Hogaboam CM, et al: TLR9 regulates the mycobacteria-elicited pulmonary granulomatous immune response in mice through DC-derived Notch ligand delta-like 4. *J Clin Invest* 2009; 119:33–46
27. Yamada Y, Nakamura H, Adachi T, et al: Elevated serum levels of thioredoxin in patients with acute exacerbation of asthma. *Immunol Lett* 2003; 86:199–205
28. Kato A, Odamaki M, Nakamura H, et al: Elevation of blood thioredoxin in hemodialysis patients with hepatitis C virus infection. *Kidney Int* 2003; 63:2262–2268
29. Callister ME, Burke-Gaffney A, Quinlan GJ, et al: Extracellular thioredoxin levels are increased in patients with acute lung injury. *Thorax* 2006; 61:521–527
30. Hofer S, Rosenhagen C, Nakamura H, et al: Thioredoxin in human and experimental sepsis. *Crit Care Med* 2009; 37:2155–2159
31. Takagi Y, Mitsui A, Nishiyama A, et al: Overexpression of thioredoxin in transgenic mice attenuates focal ischemic brain damage. *Proc Natl Acad Sci U S A* 1999; 96:4131–4136
32. Shioji K, Kishimoto C, Nakamura H, et al: Overexpression of thioredoxin-1 in transgenic mice attenuates Adriamycin-induced cardiotoxicity. *Circulation* 2002; 106:1403–1409
33. Kasuno K, Nakamura H, Ono T, et al: Protective roles of thioredoxin, a redox-regulating protein, in renal ischemia/reperfusion injury. *Kidney Int* 2003; 64:1273–1282
34. Ohashi S, Nishio A, Nakamura H, et al: Protective roles of redox-active protein thioredoxin-1 for severe acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2006; 290:G772–G781
35. Kaimul Ahsan M, Nakamura H, Tanito M, et al: Thioredoxin-1 suppresses lung injury and apoptosis induced by diesel exhaust particles (DEP) by scavenging reactive oxygen species and by inhibiting DEP-induced downregulation of Akt. *Free Radic Biol Med* 2005; 39:1549–1559
36. Sato A, Hoshino Y, Hara T, et al: Thioredoxin-1 ameliorates cigarette smoke-induced lung inflammation and emphysema in mice. *J Pharmacol Exp Ther* 2008; 325:380–388
37. Ichiki H, Hoshino T, Kinoshita T, et al: Thioredoxin suppresses airway hyperresponsiveness and airway inflammation in asthma. *Biochem Biophys Res Commun* 2005; 334:1141–1148
38. Ueda S, Nakamura T, Yamada A, et al: Recombinant human thioredoxin suppresses lipopolysaccharide-induced bronchoalveolar neutrophil infiltration in rat. *Life Sci* 2006; 79:1170–1177
39. Sato M, Hosoya M, Wright PF: Differences in serum cytokine levels between influenza virus A and B infections in children. *Cytokine* 2009; 47:65–68
40. Hagau N, Slavcovici A, Gonganau DN, et al: Clinical aspects and cytokine response in severe H1N1 influenza A virus infection. *Crit Care* 2010; 14:R203
41. Atkuri KR, Mantovani JJ, Herzenberg LA, et al: N-Acetylcysteine–a safe antidote for cysteine/glutathione deficiency. *Curr Opin Pharmacol* 2007; 7:355–359
42. Cho YS, Oh SY, Zhu Z: Tyrosine phosphatase SHP-1 in oxidative stress and development of allergic airway inflammation. *Am J Respir Cell Mol Biol* 2008; 39:412–419
43. Ghezzi P, Ungheri D: Synergistic combination of N-acetylcysteine and ribavirin to protect from lethal influenza viral infection in a mouse model. *Int J Immunopathol Pharmacol* 2004; 17:99–102
44. Garozzo A, Tempera G, Ungheri D, et al: N-acetylcysteine synergizes with oseltamivir in protecting mice from lethal influenza infection. *Int J Immunopathol Pharmacol* 2007; 20:349–354
45. Matsui M, Oshima M, Oshima H, et al: Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev Biol* 1996; 178:179–185
46. Callister ME, Burke-Gaffney A, Quinlan GJ, et al: Persistently low plasma thioredoxin is associated with meningococcal septic shock in children. *Intensive Care Med* 2007; 33:364–367
47. Leaver SK, MacCallum NS, Pingle V, et al: Increased plasma thioredoxin levels in patients with sepsis: Positive association with macrophage migration inhibitory factor. *Intensive Care Med* 2010; 36:336–341
48. Burke-Gaffney A, Callister ME, Nakamura H: Thioredoxin: Friend or foe in human disease? *Trends Pharmacol Sci* 2005; 26:398–404
49. Tao L, Gao E, Bryan NS, et al: Cardioprotective effects of thioredoxin in myocardial ischemia and reperfusion: Role of S-nitrosation [corrected]. *Proc Natl Acad Sci U S A* 2004; 101:11471–11476
50. Todoroki Y, Tsukahara H, Ohshima Y, et al: Concentrations of thioredoxin, a redox-regulating protein, in umbilical cord blood and breast milk. *Free Radic Res* 2005; 39:291–297
51. Ohta N, Tsukahara H, Ohshima Y, et al: Nitric oxide metabolites and adrenomedullin in human breast milk. *Early Hum Dev* 2004; 78:61–65

Journal of Clinical Virology 56 (2013) 25–30

Contents lists available at SciVerse ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Severe form of encephalopathy associated with 2009 pandemic influenza A (H1N1) in Japan

Akihisa Okumura[a,*], Satoshi Nakagawa[b], Hisashi Kawashima[c], Shinichiro Morichi[c], Takashi Muguruma[b], Osamu Saito[b], Jun-ichi Fujimoto[b], Chiaki Toida[b], Shuji Kuga[b], Toshihiro Imamura[b], Toshiaki Shimizu[a], Naomi Kondo[d], Tsuneo Morishima[e]

[a] Department of Pediatrics, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[b] Department of Intensive Care, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
[c] Department of Pediatrics, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-0022, Japan
[d] Department of Pediatrics, Gifu University Graduate School of Medicine, 1-1 Yanaido, Gifu 501-1194, Japan
[e] Department of Pediatrics, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan

ARTICLE INFO

Article history:
Received 21 March 2012
Received in revised form 8 October 2012
Accepted 10 October 2012

Keywords:
2009 pandemic influenza A (H1N1)
Preexisting conditions
Acute encephalopathy
Pediatric death

ABSTRACT

Background: Every year, an estimated 200–500 children in Japan develop influenza-associated encephalopathy (IAE), and 10–30% of these children die.
Objective: To clarify the clinical features of a severe form of acute encephalopathy seen with 2009 pandemic influenza A (H1N1).
Study design: This retrospective survey examined 20 children with acute encephalopathy associated with the 2009 pandemic influenza A (H1N1) who died or were in a prolonged deep coma with a flat electroencephalogram tracing and loss of spontaneous respiration. We obtained demographic, clinical, laboratory, and neuroimaging data through interviews with the attending physicians and chart reviews.
Results: Subjects were 13 boys and seven girls. Their median age was 45 (range 11–200) months. Five patients had one or more pre-existing conditions. Acute encephalopathy developed within 2 days after influenza onset in 16 patients. As the initial neurological symptom, delirious behavior was seen in six children, and brief seizures in six. Eighteen patients were comatose within 6 h of the onset of encephalopathy. Marked brain edema on computed tomography (CT) was seen in all but one patient. Brainstem lesions on CT were recognized in 12 patients. Sixteen patients died 0–45 (median 2.5) days after the onset of acute encephalopathy, and the others remained in deep comas without spontaneous respiration.
Conclusions: The clinical course of the patients was characterized by an onset with mild neurological symptoms and rapid deterioration of consciousness into coma. Head CT revealed marked cerebral edema, often associated with brainstem lesions.

1. Background

Every year, an estimated 200–500 children in Japan suffer from influenza-associated encephalopathy (IAE), and 10–30% of these children die of this severe neurological complication.[1] Pediatricians and pediatric neurologists in Japan are very concerned about IAE every winter. However, no reports have described the full details of severe IAE.

An influenza pandemic involving a novel strain of influenza A (H1N1) virus (A/H1N1 pdm 2009) emerged in 2009. Several countries reported increases in severe respiratory illnesses with A/H1N1 pdmn,[2–6] together with associated pediatric deaths.[7–11] An association of encephalopathy or severe neurological complications with pandemic influenza 2009 A/H1N1 (pH1N1) has also been reported worldwide.[12–29]

2. Objectives

The Ministry of Health, Labor and Welfare of Japan (MHLW) mandated that all medical professionals report deaths associated with pH1N1 during the 2009 pandemic. A study group funded by MHLW surveyed the data on pediatric death and found that acute encephalopathy was the one of the leading causes of pediatric death.[30] Additionally, the study group on influenza-associated

Abbreviations: MHLW, the Ministry of Health Labor and Welfare of Japan; IAE, influenza-associated encephalopathy; pH1N1, 2009 pandemic influenza A (H1N1); NIID, National Institute of Infectious Diseases in Japan; ANE, acute necrotizing encephalopathy.

* Corresponding author. Tel.: +81 3 3813 3111; fax: +81 3 5800 1580.
E-mail address: okumura@juntendo.ac.jp (A. Okumura).

1386-6532/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jcv.2012.10.007

encephalopathy conducted a questionnaire survey of children with acute encephalopathy associated with pH1N1. These studies gave us the opportunity to determine the precise features of the severe form of acute encephalopathy associated with pH1N1. This information will be also useful for understanding the deaths of children with seasonal IAE. We report the clinical features of the severe form of acute encephalopathy associated with pH1N1.

3. Study design

The study subjects were obtained from two databases. One, compiled by the study group examining pediatric deaths associated with pH1N1, included 41 fatalities younger than 20-years old, as described elsewhere.[30] By consensus, acute encephalopathy was considered to be the cause of death in 15 of the children. The second database was the results of a questionnaire survey conducted by the study group on influenza-associated encephalopathy. Every local health care center in Japan received a questionnaire inquiring about children younger than 16 years of age with pH1N1-associated encephalopathy. From both databases, we collected the patients who had pH1N1 infections from May 2009 through March 2010. According to the MHLW, approximately 20 million people were presumed to be infected by A/H1N1 pdm 2009 during the study period, and a half of them were children younger than 15 years.

The following information was collected: age, sex, virus types, history, flu vaccination record, neurological symptoms at onset, laboratory data, electroencephalogram and neuroimaging findings, treatment, clinical course, and outcome. After eliminating duplicated patients, 190 patients with acute encephalopathy associated with pH1N1 infection were identified in the combined database.

To clarify the clinical features of the severe form of pH1N1-associated encephalopathy, we investigated the children who died or were in a continuous deep coma with a flat electroencephalogram tracing (maximum amplitude <5 μV) and loss of spontaneous respiration necessitating intensive life support with mechanical ventilation. Ultimately, we identified 16 children who died and four in prolonged deep comas. These 20 children were the subjects of this study.

Acute encephalopathy was defined as at least one of the following: (1) an altered mental state without profound respiratory or cardiac failure and (2) neuroimaging findings consistent with encephalopathy, such as marked brain edema, focal lesions, and a blurred gray–white matter junction. The onset of the 2009 pandemic flu was considered the time at which a fever >38 °C was first recorded. The study group re-evaluated the radiographic data, including computed tomography (CT) and magnetic resonance imaging (MRI) of the head. Infection with A/H1N1 pdm 2009 was confirmed in all but one patient by testing nasal swabs or aspirates from the nose, throat, or tracheal tube using real-time reverse transcriptase polymerase chain reaction assays at local public health laboratories or the National Institute of Infectious Diseases in Japan (NIID) according to the protocol recommended by NIID.

This study was supported by the MHLW of Japan. As this study was considered to be a public health activity, approval from an ethics committee or institutional review boards at participating hospitals and informed consent were not required. Anonymous data were collected retrospectively and were kept confidential.

4. Results

A representative patient report is shown below.

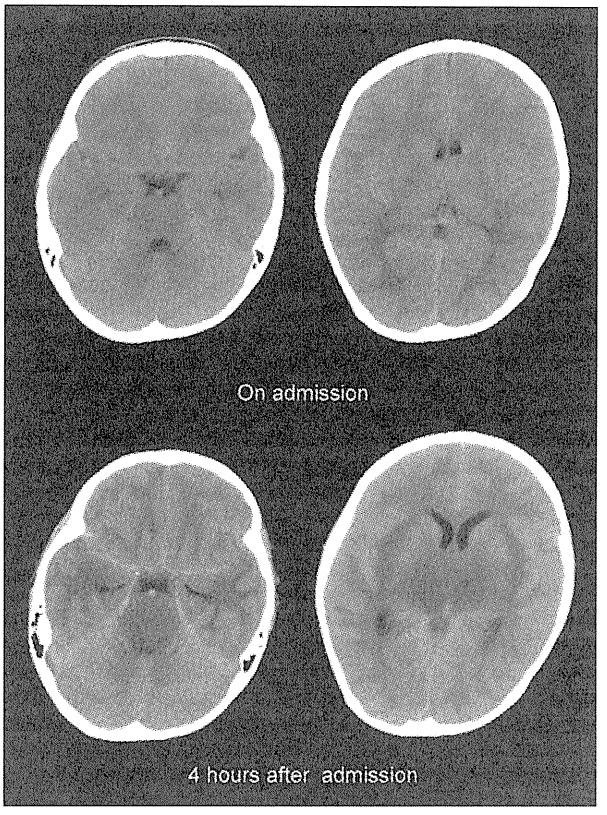

Fig. 1. Head CT findings (Patient 2). (Top) The head CT on admission shows mild low-density lesions in the brainstem (left), mild blurring of the gray–white matter junction, and mild narrowing of the extra-axial spaces, suggesting mild brain edema (right). (Bottom) The head CT 4 h after admission shows marked low-density lesions and swelling in the brainstem (left), evident blurring of the gray–white matter junction, and marked low densities in the bilateral thalami (right). Narrowing of the extra-axial spaces and fourth ventricle are also seen.

4.1. Patient report

A 5-year-old boy without pre-existing medical conditions (Patient 2) developed pyrexia, rhinorrhea, and a cough. The next morning, he was diagnosed with influenza A using a rapid antigen test and was given oseltamivir once. A few hours later, he seemed delirious, saying things such as "something is flying." He was admitted to a tertiary care hospital 3 h after the onset of the delirious behavior. On admission, mildly reduced responsiveness was noted. Otherwise, no neurological abnormalities were seen. Head CT on admission showed mild brain edema, with low-density lesions in the brainstem (Fig. 1). The gray–white matter border was blurred somewhat. After admission, steroid pulse therapy and intravenous glycerol were started immediately with supportive treatment. However, his consciousness deteriorated progressively to a deep coma in a few hours. He had two brief generalized convulsions that were controlled after a continuous midazolam infusion. Head CT 4 h after admission revealed marked brain edema with blurred gray–white matter differentiation (Fig. 1). Marked low-density lesions were observed in the brainstem and bilateral thalami. Although artificial ventilation and catecholamine administration were started, the EEG the next morning showed a flat trace. The patient died 3 days after admission.

Table 1
Demographic features.

Patient	Sex	Age (m)	Preexisting conditions	History of FS	Interval between Flu onset and AE (days)	Drugs before the onset of AE			Outcome
						Antiviral agents	Acetaminophen	Theophylline	
1	M	89	None	No	1	None	Unknown	No	Death
2	M	62	None	No	1	Oseltamivir	No	No	Death
3	M	52	None	No	2	None	No	No	Death
4	M	200	Asthma	No	1	Zanamivir	Yes	No	Death
5	F	98	Preterm	No	1	None	Yes	No	Death
6	M	121	Mild developmental delay, epilepsy	Yes	1	None	Yes	No	Death
7	F	101	None	No	1	Zanamivir	No	No	Death
8	F	43	None	No	1	None	No	No	Death
9	M	17	Preterm, asthma, mild developmental delay	Yes	1	Oseltamivir	Yes	No	Death
10	F	46	Asthma, mild developmental delay	No	1	Oseltamivir	Yes	No	Death
11	M	18	None	No	0	None	No	No	Death
12	M	53	None	No	1	Oseltamivir	Yes	No	Death
13	M	40	None	Yes	0	None	No	No	Death
14	M	107	None	No	2	Oseltamivir	No	No	Death
15	F	89	None	No	1	Oseltamivir	Yes	No	Death
16	M	21	None	No	5	Oseltamivir	Unknown	No	Death
17	F	65	None	No	0	None	No	No	Prolonged coma
18	M	11	None	No	2	None	No	No	Prolonged coma
19	F	121	None	No	1	Zanamivir	Yes	No	Prolonged coma
20	M	27	None	No	1	None	No	No	Prolonged coma

FS, febrile seizures; AE, acute encephalopathy.

4.2. Demographic data

The demographic data are summarized in Table 1. The subjects were 13 boys and seven girls. Their median age was 45 (range 11–200) months, and 10 patients were 0–4 years of age. Five patients had one or more pre-existing conditions (Table 1): asthma in three, mild developmental delay in three, and epilepsy in one. Three had histories of febrile seizures. Information on influenza vaccination was available for 17 children; three patients had had one injection of vaccine against A/H1N1 pdm 2009. A diagnosis of influenza using a rapid antigen test was made within 2 days after the onset of fever in all patients. Before the development of acute encephalopathy, seven patients received oseltamivir, and three received zanamivir. Acetaminophen was administered in eight of 18 patients for whom information was available. No patient had taken theophylline or salicylate. Chest radiographs were obtained for all patients and showed mild infiltration in four. However, no severe respiratory distress or desaturation was observed in any patient.

4.3. Acute encephalopathy

Acute encephalopathy developed on the day of influenza onset in three patients and the next day in 13 (Table 1). The neurological symptoms are summarized in Table 2. The initial neurological symptom was delirious behavior in six patients, a single brief seizure in six, status epilepticus in three, mildly altered mental status in two, coma in two, and tremulous movement in one. Thirteen patients had no reduction or a mild reduction in consciousness at onset. However, consciousness deteriorated rapidly in most patients. Coma was seen within 6 h after the initial neurological symptom in 18 patients. All patients were in a coma within 12 h after the onset of encephalopathy. Although status epilepticus was seen in four children, the seizures were treated successfully with intravenous antiepileptic drugs such as midazolam and phenobarbital.

Delirious behavior was noted in seven patients. In six, the delirious behavior was the initial neurological symptom of acute encephalopathy and was followed by altered mental status. The delirious behavior included incoherent and meaningless speech, loss of emotional control, and disorientation. The duration of the delirious behavior was typically 30 min.

4.4. Brain computed tomography

Brain CT was performed in all but one patient (Table 3). In 14 patients, brain CT was obtained within 3 h of admission, and nine of these patients had various degrees of brain edema. In this group, abnormal low densities were observed in the brainstem in seven patients, in the thalamus in one, and in the basal ganglia in one. The brain CT within 3 h of admission was unremarkable in five patients (Patients 9, 14, and 18–20). In four of these patients, subsequent CT revealed marked brain edema with or without focal lesions in the brainstem or basal ganglia. A follow-up CT could not be obtained in Patient 14 because of rapid systemic deterioration.

In four patients, the initial CT was performed 12–20 h after admission. All showed marked brain edema with blurred gray–white matter differentiation. Low densities were seen in the brainstem in three patients, in the basal ganglia in two, and in the thalamus in two. In Patient 6, head CT was done as a series of autopsy images. Marked brain edema was seen in this patient.

Brainstem lesions on CT were recognized in 12 patients. Of the six patients with delirious behavior as the initial neurological symptom, five had brainstem lesions. Thalamic and basal ganglia lesions were seen in five and three patients, respectively.

4.5. Treatment and outcome

Steroid pulse therapy was administered in 18 patients, and intravenous dexamethasone was administered in one. Intravenous immunoglobulin was administered in 11 cases, selective or systemic hypothermia in eight, edaravone in six, continuous hemodialysis filtration in 2, and plasma exchange in two. However, no patient responded well to these intensive treatments.

Sixteen patients died 0–45 (median 2.5) days after the onset of acute encephalopathy. Nine children died within 3 days of the onset of acute encephalopathy. Four patients remained in deep comas without spontaneous respiration for several months after the onset