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Antiviral activity of hypothiocyanite produced by lactoperoxidase against influenza A and B viruses and mode of its antiviral action

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Summary. – Hypothiocyanite ($OSCN^-$) is a natural component of human saliva and is produced by the lactoperoxidase (LPO)/thiocyanate/hydrogen peroxide (H_2O_2) system. $OSCN^-$ has been previously shown to exhibit antiviral activity against influenza viruses (IFV) A/H1N1/2009 and A/H1N2/2009 *in vitro* as well as antimicrobial and antifungal activities. We elucidated the antiviral activity of $OSCN^-$ against both IFV types A and B and the mode of its antiviral action. $OSCN^-$ was produced constantly at 900 ± 200 μmol/l in Na_3PO_4 buffer solution containing NaSCN and LPO in the presence of H_2O_2 as an original $OSCN^-$ solution. In a plaque reduction assay, IFV A/PR/8/34 (H1N1), A/Fukushima/13/43 (H3N2), B/Singapore/222/97, and B/Fukushima/15/93 were exposed to various concentrations of $OSCN^-$ for 0 to 30 min before adsorption to MDCK cells, and plaque formation was examined. $OSCN^-$ exhibited significant similar antiviral activities against all four viruses without cytotoxicity, and the EC_{50} values for them were from 57 ± 16 to 148 ± 27 μmol/l regardless of the exposure times. The exposure of MDCK cells to $OSCN^-$ before viral adsorption did not affect its anti-IFV activity (EC_{50}: more than 450 μmol/l), but the exposure after viral adsorption affected it moderately (EC_{50}: 380 ± 40 μmol/l). Moreover, the exposure of virus particles to $OSCN^-$ at 450 μmol/l did not affect the hemagglutinin activity of IFV in hemagglutination inhibition assay. These results suggest that the attachment of $OSCN^-$ to the viral envelope critically contributes to the mode of antiviral action of $OSCN^-$ without interfering with viral adsorption.

Keywords: hypothiocyanite; influenza virus type A; influenza virus type B; lactoperoxidase; antiviral activity

Introduction

Hypothiocyanite ($OSCN^-$) is a natural component of human saliva and a major oxidation product of the lactoperoxidase (LPO)/thiocyanate (SCN^-)/hydrogen peroxide (H_2O_2) reaction (Aune and Thomas, 1977; Wijkstrom-Frei *et al.*, 2003). This reaction is referred to as the LPO system. $OSCN^-$ produced by the LPO system inhibits bacterial metabolism by the reaction with bacterial sulfhydryls (Thomas and Aune, 1978; Thomas *et al.*, 1983). Shin *et al.* (2011) showed that ingestion of a tablet containing LPO reduces the volume of the oral bacteria that cause oral malodor and periodontal disease. Moreover, several studies have reported that $OSCN^-$ possesses inhibitory properties against fungi such as *Candida albicans* and viruses such as influenza virus (IFV), herpes simplex virus type 1, respiratory syncytial virus, echovirus 11, and human immunodeficiency virus (Pourtois *et al.*, 1990; Lenander-Lumikari, 1992; Mikola *et al.*, 1995; Cegolon *et al.*, 2014; Gingerich *et al.*, 2016). Therefore, $OSCN^-$ produced by the LPO system surely contributes to host defense against infections.

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Abbreviations: BSA = bovine serum albumin; DTNA = 5,5'-dithiobis-2-nitrobenzoic acid; EC_{50} = effective concentrations for 50% plaque reduction; HAI = hemagglutination inhibition assay; H_2O_2 = hydrogen peroxide; IFV = influenza virus; LPO = lactoperoxidase; $OSCN^-$ = hypothiocyanite; PFU = plaque forming unit; RBC = red blood cell; SCN^- = thiocyanate; SD = standard deviation; TNA = 5-thio-2-nitrobenzoic acid

IFV is a respiratory RNA virus enclosed by a lipid envelope that embeds two glycoproteins, hemagglutinin and neuraminidase. These glycoproteins assist virus adsorption to cells and virus budding during infection. IFV types A (H1N1, H1N2, and H3N2 subtypes) and B commonly infect humans and cause seasonal epidemics of disease (Steel and Lowen, 2014). Especially IFV type A causes severe pandemics involving an unpredictable morbidity and mortality in humans (Simonsen, 1999; Thompson *et al.*, 2004). Therefore, prophylaxis of IFV infection is important (Aiello *et al.*, 2010; World Health Organization, 2017). In a recent study, Gingerich *et al.* (2016) indicated that $OSCN^-$ formation by the LPO system inactivates the H1N2 subtype of IFV type A. Moreover, $OSCN^-$ has a virucidal effect against pandemic IFV (A/H1N1/2009) *in vitro* (Cegolon *et al.*, 2014). However, the antiviral effects of $OSCN^-$ on the H3N2 subtype of IFV type A and type B still remain unclear.

In the present study, we compared the antiviral activities of $OSCN^-$ against IFV types A and B and elucidated the mode of its antiviral action. We found that $OSCN^-$ exhibits similar antiviral activity against both IFV types A and B, and that the attachment of $OSCN^-$ to the viral envelope without interfering with viral adsorption to host cells critically contributes to the mode of antiviral action of $OSCN^-$.

Materials and Methods

Viruses and cells. IFV A/PR/8/34 (H1N1), A/Fukushima/13/43 (H3N2), B/Singapore/222/97, and B/Fukushima/15/93 were propagated in Madin-Darby canine kidney (MDCK) cells as described previously (Sawamura *et al.*, 2010). The infected cultures were frozen and thawed three times and then centrifuged at 880 x *g* for 15 min. The supernatants were stored at -80°C as virus stock solution until use. MDCK cells were grown and maintained in Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 8% and 2%, respectively, heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., South Logan, Utah, USA) (Kurokawa *et al.*, 1990).

$OSCN^-$ preparation by LPO system. $OSCN^-$ was enzymatically generated by the LPO system (Lloyd *et al.*, 2008). In the system, 50 mmol/l Na_3PO_4 buffer solution (pH 6.6, Nacalai Tesque, Inc., Kyoto, Japan) containing 7.5 mmol/l NaSCN (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1.5 µmol/l LPO (Morinaga Milk Industry Co., Ltd., Tokyo, Japan) was used as a reaction solution that is negative control without H_2O_2. $OSCN^-$ was generated in the reaction solution by the addition of H_2O_2 (Wako Pure Chemical Industries, Ltd.) to make a final concentration of 3.75 mmol/l in a volume of 40 ml for 15 min at room temperature. Then, catalase (1.0 ml, 140 U/ml, Wako Pure Chemical Industries) was added to remove unreacted H_2O_2, and the enzyme reaction mixture was centrifuged in filter tubes (Amicon Ultra Centrifugal Filters Ultracel-10K, Merck Millipore Ltd., County Cork, Ireland) at 2,000 x *g* for 5 min at 4°C. The filtered solution was used as an original $OSCN^-$ solution.

Measurement of $OSCN^-$ concentration. The $OSCN^-$ concentration produced in the reaction solution was measured in the presence of 5,5'-dithiobis-2-nitrobenzoic acid (DTNA, Nacalai Tesque) as reported by Tenovuo *et al.* (1985). The original prepared $OSCN^-$ solution or the reaction solution of the LPO system (as a reference) (25 µl) was mixed with 1.975 ml of 49 mmol/l Tris buffer solution (pH 8.0, Nacalai Tesque) containing 32 µmol/l DTNA, 30 µmol/l 2-mercaptoethanol (2-ME, Wako Pure Chemical Industries), and distilled water. The absorbance of 5-thio-2-nitrobenzoic acid (TNA) was measured at 412 nm within 30 min after the mixing to determine the concentration of $OSCN^-$, because TNA in the mixture is produced from DTNA by 2-ME, and 2 moles of TNA react with 1 mole of $OSCN^-$ to produce DTNA again. The $OSCN^-$ concentration was calculated from the measured absorbance using a molar absorbance coefficient of 14,150 $(mol/l)^{-1}cm^{-1}$ of TNA according to the following formula (Tenovuo *et al.*, 1985):

$OSCN^-$ (µmol/l) = (A_{412} × 1/14,150 × 1/2 × 2,000/25) mol/l × 10^6 where A_{412} = (reference absorbance) – (original $OSCN^-$ solution absorbance); 14,150 = the molar absorbance coefficient of TNA; 2 = correction factor (2 moles TNA requires 1 mole $OSCN^-$ to produce 1 mole DTNA); 2,000 = total volume (µl); 25 = sample volume (µl)

Stability of $OSCN^-$. To examine the stability of $OSCN^-$ produced by addition of H_2O_2 in original $OSCN^-$ solution, we assessed the change of $OSCN^-$ concentrations in the mixture of equal parts of the original $OSCN^-$ solution and phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma-Aldrich Corp., St. Louis, MO, USA), which was used to dilute the original virus solution as described below. The $OSCN^-$ concentrations in the mixture were measured in the presence of DTNA at 0, 15, 30, 45, 60, 75 and 90 min after the mixing at room temperature as described above. As a reference, we used the mixture of equal parts of the reaction solution without H_2O_2 and PBS containing 1% BSA. The $OSCN^-$ concentrations were calculated as described above.

Plaque reduction assay. We examined the antiviral activity of $OSCN^-$ against IFV A/PR/8/34, A/Fukushima/13/43, B/Singapore/222/97, and B/Fukushima/15/93 by a plaque reduction assay using MDCK cells. The $OSCN^-$ concentrations produced were constantly 900 ± 200 µmol/l in the original $OSCN^-$ solution. The original prepared $OSCN^-$ solution was serially diluted 2-fold with the reaction solution of the LPO system, and each dilution was mixed with an equal volume of virus solution at 200 plaque-forming units (PFU)/0.2 ml prepared from virus stock solution by the addition of PBS containing 1% BSA. The mixtures were incubated for 0, 15, and 30 min at room temperature. Then, 0.2 ml of each mixture (100 PFU) was added to confluent MDCK cells grown in 60 mm plastic dishes, and virus was adsorbed to MDCK cells at room temperature for 1 h. The inoculum was removed, and the cells were overlaid with 5 ml of a nutrient agarose (0.8 %) medium for a plaque assay and maintained in a humidified atmosphere containing 5% CO_2 for 3 to 4 days as described previously (Sawamura *et al.*,

2010). The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue. The number of plaques was counted, and the effective concentrations for 50% plaque reduction (EC_{50}) were determined from a curve relating the plaque number to the concentrations of $OSCN^-$ solutions (Shimizu *et al.*, 2008).

MDCK cells exposure to $OSCN^-$ before and after virus adsorption. We examined the effects of exposure of MDCK cells to $OSCN^-$ before and after the adsorption of IFV A/PR/8/34 to MDCK cells. The original prepared $OSCN^-$ solution was serially diluted 2-fold, and each dilution was mixed with an equal volume of PBS containing 1% BSA. The highest concentration of $OSCN^-$ in the mixture was 450 μmol/l (as a half concentration of the original $OSCN^-$ solution). To examine the effect of exposure of MDCK cells to $OSCN^-$ before viral adsorption, 0.2 ml aliquots of $OSCN^-$ dilutions were added to confluent MDCK cells in 60 mm plastic dishes and incubated for 1 h at room temperature. The cells were rinsed with PBS and inoculated with 100 PFU/0.2 ml of IFV A/PR/8/34 diluted from the virus stock solution by PBS containing 1% BSA. After adsorption at room temperature for 1 h, a plaque assay was performed as described before. On the other hand, to examine the effect of $OSCN^-$ exposure after viral adsorption, confluent MDCK cells in 60 mm plastic dishes were incubated with 100 PFU/0.2 ml of IFV A/PR/8/34 for 1 h at 4°C to avoid virus entry into the cells. The cells were rinsed with cold PBS, and 0.2 ml of the mixtures of diluted $OSCN^-$ solutions or the reaction solution of the LPO system (as a control) was added to the infected cells. After incubation of 1 h at room temperature, a plaque assay was performed as described before. After fixing and staining the infected cells, the number of plaques was counted, and EC_{50} values were determined as described before.

Cytotoxicity assay. The cytotoxicity of $OSCN^-$ toward IFV-infected MDCK cells was evaluated by a trypan blue exclusion test as described previously (Tsurita *et al.*, 2001). Briefly, MDCK cells were seeded at 3.5×10^6 cells in a 60 mm dish and grown with 8% FBS-EMEM at 37°C for 3 days. The $OSCN^-$ concentration in the original $OSCN^-$ solution was 900 ± 200 μmol/l, which was the highest concentration that could be produced in our LPO system. The original $OSCN^-$ solution or the reaction solution of the LPO system as a control was mixed with an equal volume of virus solution (IFV A/PR/8/34 at 200 PFU/0.2 ml) as described before. The concentration of $OSCN^-$ in the mixture prepared was 450 μmol/l. Immediately after mixing, 0.2 ml of the mixture (100 PFU/0.2 ml) was added to MDCK cells grown in 60 mm plastic dishes and virus was adsorbed to MDCK cells at room temperature for 1 h as described before. The cells were rinsed twice with PBS and treated with trypsin, and then the number of viable cells was examined by the trypan blue exclusion test. The cytotoxicity of $OSCN^-$ was determined from the ratio of the number of viable cells to total cells.

Hemagglutination assay and hemagglutination inhibition assay (HAI). The effect of $OSCN^-$ on virus-induced hemagglutinin activity was evaluated. Viral stock solutions of IFV A/PR/8/34, A/Fukushima/13/43, B/Singapore/222/97, and B/Fukushima/15/93 were serially diluted 2-fold with saline in 96-well V-bottom plates. Each diluted viral solution was mixed with an equal volume of a 1% suspension of guinea pig red blood cells (RBC) and incubated at 4°C. After 60 min, hemagglutination titers were determined. The hemagglutination titer of the original IFV stock solution of A/PR/8/34 was 256 and those of A/Fukushima/13/43, B/Singapore/222/97, and B/Fukushima/15/93 were 512. For the HAI, four hemagglutinin viral solutions of the four viral strains were prepared. The HAI was performed in 96-well V-bottom plates as described previously (Reading *et al.*, 2008; Chan *et al.*, 2011). Briefly, serial 2-fold dilutions of the original $OSCN^-$ solution or the reaction solution of the LPO system (as a control) was mixed with each of four hemagglutinin viral solutions and incubated at room temperature for 30 min. Then, a 1% guinea pig RBC suspension was added to the mixtures and incubated at 4°C for 60 min. Finally, hemagglutination inhibition titers in the presence or absence of $OSCN^-$ were compared.

Statistical analysis. All data are expressed as means ± standard deviation (SD). Differences between or among the individual groups were statistically evaluated by the Mann-Whitney test or ANOVA with the Kruskal-Wallis test, respectively, using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). A *P*-value below 0.05 was considered to indicate statistical significance (n indicates the number of samples).

Results

Antiviral activity of $OSCN^-$ against IFV type A and B

The antiviral activity of $OSCN^-$ against IFV A/PR/8/34, A/Fukushima/13/43, B/Singapore/222/97, and B/Fukushima/15/93 was examined by a plaque reduction assay using MDCK cells. The $OSCN^-$ produced by H_2O_2 was stable in the mixture of original $OSCN^-$ solution and PBS containing 1% BSA used as the dilution buffer of virus stock solution at least up to 90 min after their mixing as shown in Fig. 1. IFV was exposed to various concentrations (less than 450 μmol/l) of $OSCN^-$ prepared from the original $OSCN^-$ solution (900 ± 200 μmol/l) at room temperature for 0, 15, and 30 min and then adsorbed to MDCK cells in the presence of $OSCN^-$ for 1 h. Fig. 2 shows a representative test of plaque formation of IFV A/Fukushima/13/43 exposed to $OSCN^-$ for 30 min. $OSCN^-$ dose-dependently decreased the number of plaques. At concentrations of $OSCN^-$ higher than 196 μmol/l, the plaque formation was inhibited by more than 98%. Table 1 shows the plaque formation of IFV types A and B strains exposed to $OSCN^-$ before and during viral adsorption. The EC_{50} values of $OSCN^-$ for A/PR/8/34, B/Singapore/222/97, and B/Fukushima/15/93 were not significantly affected by the exposure times of 0, 15, and 30 min before viral adsorption. The EC_{50} values at 0 and 30 min of the four IFV strains were similar, and the differences were not significant. At 15 min, there were no significant differences in the EC_{50} values for A/PR/8/34, B/Singapore/222/97, and B/Fuku-

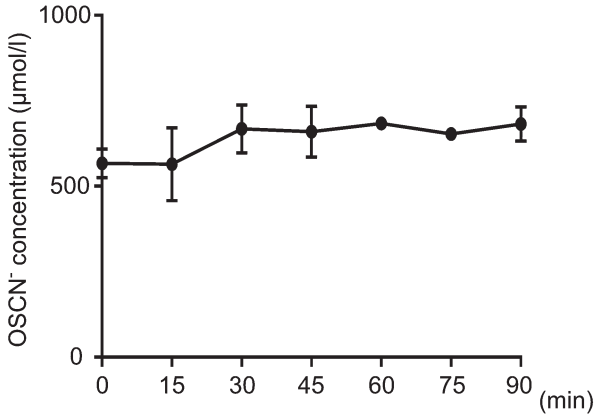

Fig. 1

Stability of prepared $OSCN^-$
The stability of $OSCN^-$ produced by H_2O_2 was examined in the mixture of original $OSCN^-$ solution and PBS containing 1% BSA used as the dilution buffer of virus stock solution as described in text. $OSCN^-$ was time-dependently stable up to 90 min ($P = 0.064$ by Kruskal-Wallis test).

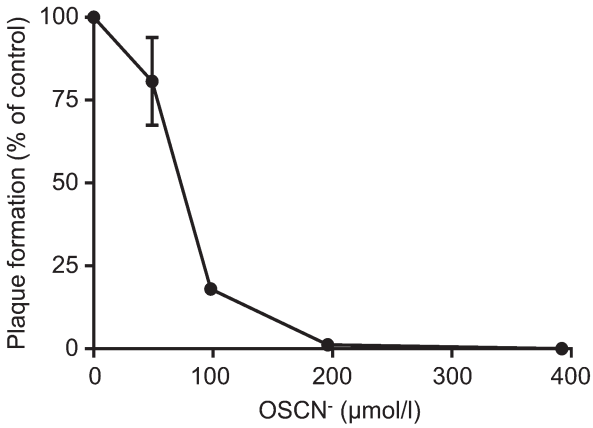

Fig. 2

Effect of $OSCN^-$ on plaque formation of IFV A/Fukushima/13/43
Antiviral activity of different concentrations of $OSCN^-$ against IFV A/Fukushima/13/43 after 30 min exposure was examined in a plaque reduction assay using MDCK cells. Mean ± SD of three independent experiments. Bar indicates SD.

shima/15/93, although the EC_{50} value (148 ± 27 μmol/l) for A/Fukushima/13/43 was significantly higher than that for B/Fukushima/15/93 but not those for A/PR/8/34 and B/Singapore/222/97. Antiviral activities of $OSCN^-$ against IFV types A and B were not markedly different. For A/Fukushima/13/43, the EC_{50} value (73 ± 1 μmol/l) of $OSCN^-$ at 30 min was significantly lower than that at 15 min but not at 0 min. For all four IFV examined, the antiviral activity of $OSCN^-$ was likely to be independent of its exposure time to IFV before viral adsorption. Thus, $OSCN^-$ exhibited similar antiviral activity against IFV types A and B, and the effect of $OSCN^-$ on viral particles was suggested to occur shortly before viral adsorption.

Effect of $OSCN^-$ treatment of MDCK cells before and after viral adsorption

Because the antiviral activities of $OSCN^-$ against IFV types A and B were similar, we used the A/PR/8/34 strain as a representative of IFV types A and B to evaluate the effects of $OSCN^-$ exposure on MDCK cells before and after viral adsorption. In both cases, MDCK cells were exposed to $OSCN^-$ for 1 h. As shown in Fig. 3, the exposure to $OSCN^-$ at 450 μmol/l before viral adsorption did not affect the plaque-forming ability of the A/PR/8/34 strain, but the exposure to $OSCN^-$ at 450 μmol/l after viral adsorption significantly reduced the plaque-forming ability. The EC_{50} values of $OSCN^-$ for MDCK exposure before and after viral adsorption were more than 450 μmol/l and 380 ± 40 μmol/l, respectively, and significantly higher than EC_{50} values in the case of exposure to $OSCN^-$ before and during viral adsorption, as shown in Table 1 (0.078 ± 0.014, 0.083 ± 0.001, and 97 ± 13 μmol/l). Exposure of MDCK cells to $OSCN^-$ before viral adsorption did not affect the antiviral activity of $OSCN^-$ but did moderately after viral adsorption.

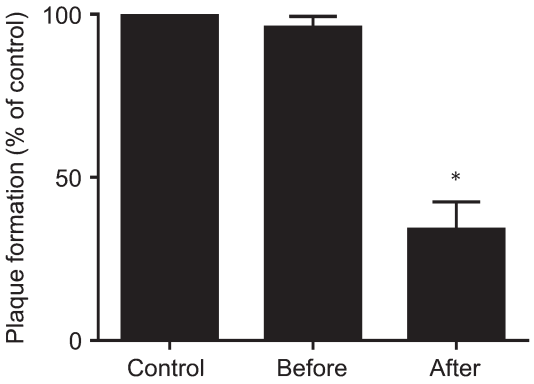

Fig. 3

Effect of $OSCN^-$ on MDCK cells before and after viral adsorption
The effect of $OSCN^-$ exposure of MDCK cells before and after adsorption of IFV A/PR/8/34 to MDCK cells was examined by a plaque reduction assay. *P <0.0001 vs. control on plaque formation ratios (%) among control, before, and after viral adsorption by the Kruskal-Wallis test (n = 6).

Cytotoxicity of $OSCN^-$ to MDCK cells

The cytotoxicity of $OSCN^-$ was examined to characterize its potential antiviral activity, as shown in Table 1. MDCK cells were exposed to $OSCN^-$ for 1 h, corresponding to the time of viral adsorption in the plaque reduction assay,

Table 1. Antiviral activity of $OSCN^-$ against IFV types A and B before and during viral adsorption to MDCK cells

Exposure time (min)	EC_{50} (μmol/l)			
	A/PR/8/34	A/Fukushima/13/43	B/Singapore/222/97	B/Fukushima/15/93
0	97 ± 13	111 ± 37	133 ± 25	88 ± 5
15	78 ± 14	148 ± 27	84 ± 12	57 ± 16*
30	83 ± 1	73 ± 1*	70 ± 15	76 ± 19

EC_{50}, effective concentrations for 50% plaque reduction. *P <0.05 vs A/Fukushima/13/43 at 15 min among the groups after 15 min exposure of IFV to $OSCN^-$ or among three different times of $OSCN^-$ exposure of A/Fukushima/13/43 by the Kruskal-Wallis test (3 independent experiments). Data are shown as means ± SD.

and the ratios of viable cells in the presence and absence of $OSCN^-$ were compared. The $OSCN^-$ concentration of 450 μmol/l was prepared from the highest concentration (900 ± 200 μmol/l) that we could produce in the LPO system and was much higher than the EC_{50} values shown in Table 1. As shown in Fig. 4, there was no significant difference in the ratios of viable cells in the presence and absence of $OSCN^-$ at 450 μmol/l. Therefore, $OSCN^-$ is potentially not cytotoxic *in vitro*.

Effect of $OSCN^-$ on hemagglutinin activity

The effect of $OSCN^-$ on hemagglutinin activity of IFV was examined to characterize the inhibitory effect of $OSCN^-$ on the adsorption of IFV to MDCK cells. As shown in Fig. 5b, hemagglutinin activities of IFV [A/PR/8/34 (H1N1), A/Fukushima/13/43 (H3N2), B/Singapore/222/97, and B/Fukushima/15/93] at four hemagglutinin units were not inhibited in the reaction solution used as a control. As shown in Fig. 5a, hemagglutinin activities of the four IFVs were not inhibited in the presence of $OSCN^-$ either, even at the highest

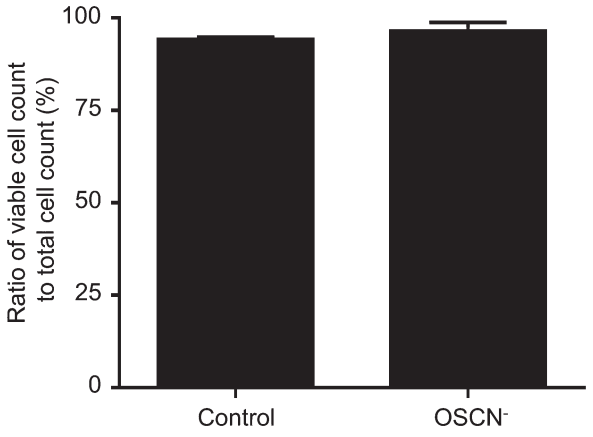

Fig. 4

Cytotoxicity of $OSCN^-$ to MDCK cells

The cytotoxicity of $OSCN^-$ to MDCK cells was assessed by trypan blue exclusion test. The ratio of viable cell counts to total cell counts was expressed as mean ± SD of 3 independent experiments. Differences between control group and $OSCN^-$ group were statistically evaluated by the Mann-Whitney test (P = 0.3429).

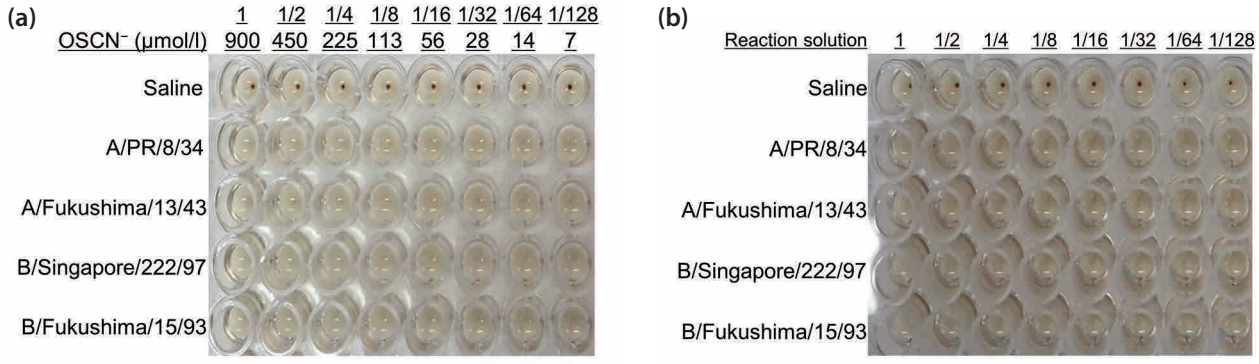

Fig. 5

Effect of $OSCN^-$ on hemagglutinin activity of IFV

The effect of $OSCN^-$ on hemagglutinin activity of IFV was evaluated by HAI. **(a)** $OSCN^-$ (900 μmol/l) or **(b)** reaction solution (as a control) was serially diluted 2-fold and added to 96-well plates. Four hemagglutinin units of each IFV strain [A/PR/8/34 (H1N1), A/Fukushima/13/43 (H3N2), B/Singapore/222/97, and B/Fukushima/15/93] were added to the 96-well plates. Then, 1% guinea pig RBC suspension was added to the mixtures as described in the Material and Methods.

concentration (450 μmol/l) of $OSCN^-$ which exhibited anti-IFV activity. $OSCN^-$ concentrations up to 450 μmol/l had no effect on hemagglutinin activity of the four IFVs examined.

Discussion

In the present study, we showed that $OSCN^-$ exhibits antiviral activity against not only IFV type A but also type B. The exposure of MDCK cells to $OSCN^-$ before IFV adsorption had no effect on the viral growth, and the exposure to virus particles did not affect hemagglutinin activity of IFV either. However, the exposure of cells following virus adsorption to $OSCN^-$ moderately affected the viral growth, and the exposure of viral particles to $OSCN^-$ before viral adsorption to host cells was significantly effective in inhibiting viral growth. Thus, the attachment of $OSCN^-$ to the components of the viral envelope without interfering with viral adsorption to host cells was suggested to contribute critically to the mode of antiviral action of $OSCN^-$.

The LPO system converts SCN^- to $OSCN^-$ in the presence of LPO and H_2O_2, and $OSCN^-$ acts as the host defense against infection. Shin *et al.* (2008) reported that the formation of $OSCN^-$ in saliva shows bactericidal activity against oral bacteria and contributes to a reduction of breath odor. $OSCN^-$ inhibits a wide variety of viruses as well as bacteria (Pourtois *et al.*, 1991; Mikola *et al.*, 1995; Shin *et al.*, 2002). In addition, recent studies suggested that $OSCN^-$ inactivates pandemic IFV (A/H1N1/2009) and IFV A/H1N2/2009 *in vitro* (Cegolon *et al.*, 2014; Gingerich *et al.*, 2016). Our results confirmed the inhibitory effect for IFV type A. However, the effect of $OSCN^-$ against type B IFV has been unknown. The present study found that $OSCN^-$ exhibited antiviral activity against both types A and B IFV *in vitro*. This is the first study demonstrating the antiviral activity of $OSCN^-$ against both IFV types A (H3N2 subtype) and B. In this study, the EC_{50} values of $OSCN^-$ for four IFV strains were determined by plaque reduction assay and 57 to 148 μmol/l. These EC_{50} values were higher than the value (2 μmol/l) for A/H1N1 2009 pandemic IFV determined by a specific reverse transcriptase real-time-PCR, which was reported by Cegolon *et al.* (2014). The plaque reduction assay is quantitatively much better way than real-time-PCR to assess the number of infective viral particles. Thus, our results would be useful and valuable to evaluate anti-influenza virus activity *in vivo*, especially in saliva containing $OSCN^-$. Antiviral activity of $OSCN^-$ against IFV was independent of the exposure time of IFV to $OSCN^-$ in the plaque reduction assay. It is probable that $OSCN^-$ reacted quickly with some component of the influenza viral envelope.

$OSCN^-$ is the final product of the LPO system and is normally present in the human body with SCN^- (Pruitt *et al.*, 1982). $OSCN^-$ is efficiently generated from SCN^- by peroxidase in the presence of H_2O_2 *in vivo* because H_2O_2 is contained in and/or generated from various kinds of foods and additives (Tenovuo *et al.*, 1985; Akagawa *et al.*, 2003). Shin *et al.* (2005) have shown that oral administration of LPO attenuated pneumonia in a murine IFV infection model. Thus, $OSCN^-$ produced by LPO *in vivo* might contribute to the alleviation of pneumonia. Previous reports showed that SCN^- and $OSCN^-$ were detected in whole saliva at concentrations of 0.5–4 mmol/l and 10–212 μmol/l, respectively (Tenovuo and Makinen, 1976; Tenovuo *et al.*, 1981; Thomas *et al.*, 1980; Pruitt *et al.*, 1983). The EC_{50} values of $OSCN^-$ for the IFV types A and B examined in this study were from 57 to 148 μmol/l. Actually, our results are within the range of $OSCN^-$ in saliva supplemented with H_2O_2 and SCN^- (Tenovuo *et al.*, 1981). No cytotoxic effect was observed at concentrations higher than the EC_{50} values. Thus, $OSCN^-$ may be an anti-IFV component *in vivo*, and the sufficient generation of $OSCN^-$ *in vivo* probably contributes to the prophylactic management of IFV infection.

When MDCK cells were exposed to $OSCN^-$ before IFV A/PR/8/34 adsorption, the plaque-forming ability of IFV was not affected. This indicates that exposure of MDCK cells to $OSCN^-$ did not inhibit the viral attachment to the cells and/or the growth in the cells. On the other hand, the exposure of virus-adsorbed MDCK cells to $OSCN^-$ reduced the plaque-forming ability of IFV A/PR/8/34 by more than 50%, when the highest concentration (450 μmol/l) of $OSCN^-$ that could be produced by the LPO system was used. It is possible that the exposure to $OSCN^-$ interfered with viral entry into MDCK cells after the attachment and/or the stages of viral replication after the entry. However, the EC_{50} value (380 ± 40 μmol/l) for $OSCN^-$ exposure after viral attachment to MDCK cells was much higher than the value (78–97 μmol/l) for A/PR/8/34 in the plaque reduction assay. In the plaque reduction assay, IFV particles were first exposed to $OSCN^-$ directly, and then IFV was adsorbed to MDCK cells in the presence of $OSCN^-$. Thus, the exposure of viral particles to $OSCN^-$ was a critical step in the plaque formation, and some component of the viral envelope associated with $OSCN^-$ might largely have contributed to the reduction of plaque formation.

Virus adsorption to cells is an initial step in IFV infection and is performed by the binding between hemagglutinin of IFV and sialic acid on the cell surface (Suzuki *et al.*, 1986; Skehel and Wiley, 2000). Exposure to $OSCN^-$ did not affect the hemagglutinin activity of IFV. $OSCN^-$ was indicated not to interfere with the binding of hemagglutinin molecules and sialic acid. Because the exposure of MDCK cells to $OSCN^-$ showed no effect on plaque formation, $OSCN^-$ did not interfere with the binding of hemagglutinin molecules and sialic acid to the surface of host cells. Therefore, it was suggested that $OSCN^-$ associated with some component on the viral envelope without interfering with viral adsorption to host cells and reduced the plaque formation of IFV.

In conclusion, our results showed that $OSCN^-$ produced by an LPO system possesses antiviral activity against both IFV types A and B. The mode of the antiviral action of $OSCN^-$ was suggested to be based on the association of $OSCN^-$ with some component on the viral envelope without interfering with viral adsorption to host cells.

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