Fucoidan inhibits parainfluenza virus type 2 infection to LLCMK₂ cells

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ABSTRACT

The effects of fucoidan and L-fucose, a fundamental major component of fucoidan, on the growth of human parainfluenza virus type 2 (hPIV-2) in LLCMK₂ cells were investigated. Fucoidan inhibited cell fusion and hemadsorption, but L-fucose only partly inhibited both. Virus RNA was not detected in the hPIV-2 infected cells cultured with fucoidan. However, L-fucose did not inhibit virus RNA synthesis. Indirect immunofluorescence study showed that virus protein synthesis was inhibited by fucoidan, but not by L-fucose. Furthermore, using a recombinant, green fluorescence protein-expressing hPIV-2, it was found that virus entry was inhibited by fucoidan, but not by L-fucose. These results suggested that fucoidan inhibited virus adsorption to the surface of the cells by binding to the cell surface and prevented infection, indicating that the sulfated polysaccharide form was important for the inhibition by fucoidan.

The inhibitory effect of sulfated polysaccharides, including fucoidan, on the replication of enveloped viruses such as herpes simplex virus, human cytomegalovirus, vesicular stomatitis virus (VSV), Sindbis virus and human immunodeficiency virus (HIV) was reported by Baba *et al.* (1). The mechanism of the inhibitory action of these compounds has been attributed to the inhibition of virus adsorption to the cells. Fucoidan is a sulfated polysaccharide and is extracted from the brown alga Okinawa mozuku *Cladosiphon okamuranus* and is effective in controlling white spot syndrome in kuruma shrimp, *Penaeus japonicus* (8).

Human parainfluenza virus type 2 (hPIV-2) is one

of the major human respiratory tract pathogens of infants and children. hPIV-2 is a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, and it possess a single-stranded nonsegmented and negative stranded RNA genome of approximately 15k bases. The gene order of hPIV-2 is 3'-(leader)-NP-V/P-M-F-HN-L-(trailer)-5'. All genes of hPIV-2 were sequenced by our group (2–6, 10), monoclonal antibodies (mAbs) were made by Tsurudome *et al.* (9), and the infectious hPIV-2 from cDNA clone was constructed by Kawano *et al.* (7).

In the present study, we investigated the effect of fucoidan on the replication of hPIV-2 in LLCMK₂ cells. Virus RNA was prepared and amplified by polymerase chain reaction (PCR). Virus protein expression was observed by indirect immunofluorescence study using mAbs against NP, F and HN proteins of hPIV-2. The inhibitory effect of fucoidan on the entry of hPIV-2 into LLCMK₂ cells was analyzed using a recombinant, green fluorescence protein (GFP)-expressing hPIV-2 (rgPIV-2).

Fucoidan (more than 80% pure: Miyako Kagaku,

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Table 1 Primers for cDNA synthesis and PCR

	Forward	Reverse
NP	ATTTCTGGGGAAGTCCAGA	GTCTAGTTTGATTGTCTGG
	(1021–1040)	(1861–1880)
F	CCCTATCCCTGAATCACAAT	TGTCACGAGACGTTACGACA
	(5821–5840)	(6661–6680)
HN	ATTTCCTGTATATGGTGGTC	GAAGTCCCCTAAAAGAGATG
	(7741–7760)	(8481–8500)

The figures show nucleotide number in all hPIV-2 genes.

Tokyo, Japan) was dissolved at 100 mg/mL in 10 mM phosphate buffered saline, pH 7.2 (PBS). After sterilization by autoclaving, it was centrifuged at 2500 rpm 10 min, and a small amount of insoluble materials were discarded. L-fucose (Nakarai Chemicals, Osaka, Japan) was dissolved at 200 mg/mL in PBS and sterilized by filtration.

Human parainfluenza virus type 2 (hPIV-2) (Toshiba strain) was used. rghPIV-2 was constructed by the method reported by Kawano *et al.* (7). There was no significant difference in the replication of the rghPIV-2 compared to that of hPIV-2 (data not shown). LLCMK₂ cells (rhesus monkey kidney cell line) were cultured in a flat-bottomed 24-well plate in 1 mL culture medium. Minimum essential medium α (MEM α) (Wako, Osaka, Japan) supplemented with 2% fetal calf serum (FCS) and 0.1 mg/mL kanamycin was used. The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. When the cells became confluent (5×10^5 cells), the medium was changed to MEM α with 0.5% FCS and 0.1 mg/mL kanamycin, fucoidan or L-fucose was added to the cells, and they were infected with hPIV-2 (300 TCID₅₀). The cell fusion and hemadsorption (Had) were observed at 4 day post infection. Had test was carried out using sheep red blood cells (SRBC). The cells were incubated with 0.4% SRBC at room temperature for 30 min, washed 4 times with PBS, and Had was observed under a light microscope for cell culture. RNA was extracted from the cells (2×10^6 cells) cultured in a flat-bottomed 6-well plate using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's method. cDNA was synthesized with 4 μ g of RNA using superscript II reverse transcriptase (Invitrogen) with forward primers for NP, F and HN genes of hPIV-2 (Table 1). PCR was done with cDNA using forward and reverse primers for NP, F and HN genes (Table 1) and Ex Taq (Takara, Shiga, Japan).

The immunofluorescence study was performed as follows. The cells were fixed with 10% formalde-

Fig. 1 Fucoidan inhibited RNA synthesis of hPIV-2, but L-fucose did not inhibit. Lane 1: marker (base pair); lanes 2, 3, and 4 : NP, F and HN of hPIV-2, respectively; lanes 5, 6, and 7 : NP, F and HN of hPIV-2 added with fucoidan; lanes 8, 9, and 10: NP, F and HN of hPIV-2 added with L-fucose.

hyde-PBS at room temperature for 15 min, washed with PBS, and incubated with mouse mAbs against NP, F and HN proteins of hPIV-2 at room temperature for 30 min. After washing with PBS, the cells were incubated with Alexa 488 conjugated second antibody to mouse IgGs (Invitrogen) at room temperature for 30 min. The cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Fucoidan (300 μ L: equivalent to 30 mg) completely inhibited both cell fusion and Had (data not shown). A lot of cell fusion and Had were observed in hPIV-2 infected cells with 20 mg L-fucose (data not shown). Even high doses of L-fucose (40 mg and 60 mg) did not completely inhibit both cell fusion and Had (data not shown): a little fusion and Had were observed dose-independently. Fucoidan and L-fucose did not disturb normal cell morphology at the concentrations used in the experiments.

Virus RNA synthesis of the cells cultured with fucoidan or L-fucose was analyzed. As shown in Fig. 1, fucoidan completely inhibited RNA synthesis. No NP, F nor HN gene of hPIV-2 was detected in the hPIV-2 infected cells cultured with fucoidan.

Fig. 2 Effect of fucoidan and L-fucose on the expression of NP, F and HN proteins of hPIV-2. The expression of NP (A), F (B) and HN (C) proteins of hPIV-2 infected cells. Fucoidan inhibited completely the expression of NP (D), F (E) and HN (F) proteins. L-fucose did not inhibit the expression of NP (G), F (H) and HN (I) proteins. Bar 25 μ m

Fig. 3 Fucoidan inhibited the entry of rg hPIV-2, but L-fucose did not. Cells infected with rg hPIV-2 (A), cells infected with rg hPIV-2 cultured with fucoidan (B), and cells infected with rg hPIV-2 with L-fucose (C). Bar 25 μ m

However, L-fucose did not inhibit virus RNA synthesis: NP, F and HN genes were detected just the same as in hPIV-2 infected positive controls (Fig. 1).

Indirect immunofluorescence study was performed to investigate the effects of fucoidan (300 μ L) and L-fucose (60 mg) on hPIV-2 protein expression. LL-CMK₂ cells were cultured with fucoidan or L-fucose, and were infected with hPIV-2. At 4 day post infection, the cells were fixed and stained with the mAbs against NP, F and HN proteins of hPIV-2. Figs. 2A, B and C show the NP, F and HN protein expression in hPIV-2 infected cells, respectively. In hPIV-2 in-

fect cells, NP, F and HN proteins were clearly observed in almost all the cells. Fucoidan completely inhibited the expression of NP (Fig. 2D), F (Fig. 2E), and HN (Fig. 2F) proteins. However, L-fucose only partly inhibited protein expression: NP, F and HN proteins were still clearly observed in a lot of cells, though the intensity was a little weaker than that of hPIV-2 infected cells (Figs. 2 G, H, and I, respectively).

These results suggested that fucoidan inhibited the entry of hPIV-2 into cells by binding to the cell surface. To confirm this possibility, rg hPIV-2 was

used. Fucoidan or L-fucose was added to the cell culture, the cells were infected with rghPIV-2 (6×10^5 TCID₅₀) and cultured for 24 h. The cells were fixed with 3.3% formaldehyde-PBS and observed under a fluorescence microscope. As shown in Fig. 3A, the cells infected with rghPIV-2 had strong fluorescence. In the samples cultured with fucoidan, no cells had fluorescence of GFP (Fig. 3B), indicating that fucoidan inhibited the entry of rghPIV-2 into cells. However, in the rghPIV-2 infected samples cultured with L-fucose, some cells had strong fluorescence (Fig. 3C), indicating that L-fucose could not inhibit the entry of rghPIV-2 into cells.

The effect of fucoidan, one of the sulfated polysaccharides, on hPIV-2 infection was analyzed in the present study, and we showed that it had an inhibitory effect. Virus RNA and protein syntheses were completely inhibited by fucoidan. In addition, it was indicated that fucoidan inhibited the entry of hPIV-2. Fucoidan is a large polymer of sulfated fucose. The effect of L-fucose, a monosaccharide, on hPIV-2 replication was analyzed. L-fucose partly inhibited cell fusion, HA and virus protein synthesis. However, L-fucose did not inhibit either virus RNA synthesis or virus entry into cells. These results indicated that the sulfated polymer form is important for the inhibitory effect of fucoidan.

Baba *et al.* reported that sulfated polysaccharides inhibited the growth of various enveloped viruses such as herpes simplex virus, VSV and HIV (1). They also showed that sulfated polysaccharides, including fucoidan, did not inhibit replication of parainfluenza virus type 3 (hPIV-3). However, in the present investigation, fucoidan inhibited replication of hPIV-2. The homology between structural proteins of hPIV-2 and those of hPIV-3 is not so high (2–6, 10). They belong to different genera, and hPIV-2 and hPIV-3 might use different molecules of the cell surface as receptors. It was also indicated by Takahashi *et al.* (8) that fucoidan inhibited the attachment of white spot syndrome virus to the cells, resulting in prevention of virus infection in kuruma shrimp. Our data are consistent with their results: it was shown that, using rghPIV-2, fucoidan could inhibit the entry of the virus into cells. Hemagglutination (HA) test of fucoidan was carried out and the result that fucoidan had HA activity (data not shown) supported the possibility that it could bind to the surface of the cells and prevent virus at-

tachment to the cells.

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