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Prevention of human enterovirus 71 infection by kappa carrageenan

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ABSTRACT

Enterovirus 71 (EV 71), the newest member of *Enteroviridae*, is notorious for its etiological role in epidemics of the hand-foot-and-mouth disease, particularly in association with fatal neurological complications in young children. Searching for new and more effective agents against EV 71 infections has never relented as corresponding vaccines or antiviral drugs remain unavailable. Sulfated polysaccharides from seaweed are known to possess a broad range of biological activities across anti-virus, anti-tumor, immunomodulation, anti-coagulation, etc. In this study, we report kappa carrageenan also has a strong and effective anti-EV 71 activity able to reduce plaque formation, prevent viral replication before or during viral adsorption, as well as inhibit EV 71-induced apoptosis. In virus binding assay, kappa carrageenan was shown able to bind EV 71 firmly, forming carrageenan-viruses complexes, whereby the virus-receptor interaction is likely disrupted. Added together, kappa carrageenan may be an ideal candidate worthwhile to develop into anti-EV 71 agents.

1. Introduction

Enterovirus 71 (EV 71), a single positive-strand RNA genome of approximately 7400 nucleotides, belongs to the genus *Enterovirus* within the family of *Picornaviridae* (Lin et al., 2003). The human enteroviruses comprise five subgroups: poliovirus, coxsackievirus A, coxsackievirus B, echovirus, and enterovirus (Li et al., 2007). A single enterovirus particle is normally composed of 60 copies of capsids, each of which is made up of four capsid proteins: VP1, VP2, VP3, and VP4. The occlusions among VP1, VP2 and VP3 form canyons where residues on the canyon surface interact with specific host's cell surface receptors. Mutations in these regions likely alter virus-receptor binding affinity as exemplified in polioviruses and rhinoviruses (Buenz and Howe, 2006; Ke et al., 2006).

The very first case of EV 71 infection was identified in the United States in 1969; subsequent outbreaks were reported in Australia, Southeast Asia, Europe, etc. from time to time (McMinn, 2002). The latest EV 71 was characterized to be of neurotropism, often resulting in a severe disease condition or even a sudden death. In 1998, Taiwan experienced a bitter and widespread outbreak of enterovirus infections, causing >400 children hospitalized and 78 lives claimed. Victims of the infections may appear several unique clinical syndromes, including hand-foot-and-mouth disease (HFMD), herpangina, aseptic meningitis and fatal encephalitis (Lin et al., 2003). The EV 71 neuropathology-dependent central nervous system (CNS) injury is considered to be the leading cause to damage neurons that make neurological destructions afterwards. Survivals from EV 71 CNS infection often suffer from neurological sequelae and delayed neurodevelopment (Chang et al., 2007). Pleconaril, a potent anti-picornavirus agent, inhibits rhinovirus and some enterovirus through interfering in the capsid-receptor binding, so as to prevent viruses from attachment to host cells as well as the subsequent uncoating of viral RNA. Unfortunately, pleconaril is clinically incompetent of neutralizing the cytopathic effect (CPE) induced by EV 71 isolates from the 1998 outbreak in Taiwan (Shia et al., 2002; Shih et al., 2003). Therefore, searching for more specific and effective anti-EV 71 agents is urgent.

Sulfated polysaccharides from seaweed are known to possess a broad range of biological activities, including anti-tumor (Mou et al., 2003), immunomodulation (Leiro et al., 2007), anti-oxidation (Qi et al., 2005), and anti-inflammation (Berteau and Mulloy, 2003). Sulfated polysaccharides have also been shown to have high antiviral activities, particularly effective against such enveloped viruses as human immunodeficiency virus (HIV), herpes simplex virus (HSV), human cytomegalovirus (HCMV), dengue virus and respiratory syncytial virus (RSV) (Damonte et al., 2004). Carrageenan is abundant water-soluble sulfated galactan in *Gigartina*, *Chondrus*, *Eucheuma*, *Hypnea*, etc. and is a major cell-wall

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component in red algae (Rhodophyta). Carrageenan has been widely used as food additives, such as emulsifier, stabilizer, and thickener (Campo et al., 2009). Carrageenan was identified with a high anti-coagulant activity (Carlucci et al., 1999; Damonte et al., 2004). Carrageenan and its depolymerized products, oligosaccharides, also carry favorable pharmacological properties, from which such products as an excipient/disintegrant, a cough-relieving agent, an anti-viral agent, a blood fat reducing agent and cholesterin were made (Shi et al., 2000). Carrageenan may also possess some anti-tumor activities (Coombe et al., 1987; Noda et al., 1989). Most excitingly, carrageenan was reported to have antiviral activities effectively against some pathogenic viruses, for example: κ-carrageenan oligosaccharides inhibiting influenza A (H1N1) virus (Wang et al., 2011); ι-carrageenan inhibiting dengue virus-2 (DENV-2) (Talarico et al., 2011), influenza A (H1N1) virus (Leibbrandt et al., 2010), and human rhinoviruses (HRV) (Grassauer et al., 2008); natural carrageenan inhibiting herpes simplex virus-1 (HSV-1) (Mateu et al., 2011); λ-carrageenan inhibiting dengue virus-2 (DENV-2) (Talarico and Damonte, 2007); carrageenan/MIV-150 (PC-815), a combination of microbicides, inhibiting hepatitis A virus (HAV) and human papillomaviruses (HPV) (Roberts et al., 2007); λ-, κ/ι- and μ/ν carrageenan inhibiting herpes simplex virus (HSV) (Carlucci et al., 1997, 1999). Whether carrageenan is capable of counteracting pathogenicity of EV 71, however, has not yet been reported. In this study, we report that carrageenan is an excellent agent inhibiting EV 71 infections as well as ameliorating the EV 71-induced cytopathic effects.

2. Materials and methods

2.1. Cells, virus and compounds

The newly identified fatal strain of EV 71 (MEL701) was isolated from an autopsied throat swab specimen of a 2-year-old female child who was infected by EV 71 and died in July, 2001 (Chen et al., 2008). Vero cells, an African green monkey kidney cell line, were cultured as described below and used for the propagation of virus. These cells were cultured in RPMI-1640 medium with 2% fetal bovine serum (FBS) at 37 °C. When cytopathic effect (CPE) was observed in 80% of the cells, culture fluid was collected and stored in −80 °C for further virus titer detection (Chen et al., 2008). Kappa carrageenan (the major component) was purchased from Sigma–Aldrich.

Kappa carrageenan was determined by high performance liquid chromatography (HPLC). The HPLC system consisted of a pump unit PU-1580 from Jasco (Tokyo, Japan), a gel-permeation chromatography (GPC) column (OHpak SB-802, 804 HQ) (Shodex, Showa Denko, Tokyo, Japan). Carrageenan (1 mg) was dissolved in 1 ml of deionized water. The mobile phase for HPLC was deionized water with a flow rate of 0.8 ml/min. Retention time was recorded using EC 2000 GPC software. Calibration curves were prepared using the standard Pullulan (molecular weights form 504–788 KDa), which was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

For simplification, carrageenan stands for κ carrageenan hereafter unless otherwise stated.

2.2. Cytotoxicity measured by MTS assay

Cytotoxicity of carrageenan was evaluated by cell viability assays. Cell viability was measured by the MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H- tetrazolium, inner salt]) (Promega, Madison, USA) method. Vero cells (ATCC CCL-81, African green monkey kidney cell line) were incubated in 96-well plates and exposed to different concentrations (1000, 100, 10, 1 μg/ml) of carrageenan in 100 μl in RPMI-1640 containing 2% FBS in sequence (Gibco, Invitrogen Corporation, CA, USA) at 37 °C for 48 h. At due time, 20 μl of the MTS solution was added into the culture, which then was incubated for another 1 h at 37 °C. Absorbance was measured by an automated plate reader (μQuant™, BIO-TEK Instrument, Inc., Winooski, VT) at absorbance 490 nm. The percentage of viability is expressed in relation to the cell alone group (treated without carrageenan). All determinations were performed three times and each in triplicate.

2.3. Plaque reduction assays

The inhibitory effect of carrageenan on virus infection was measured by the plaque reduction assay. Different concentrations (1000, 100, 10 μg/ml) of carrageenan in 1 ml RPMI-1640 medium were mixed with an equal volume of infectious EV 71 in RPMI-1640 medium. The mixture was incubated at 37 °C for 1 h. At due course, the mixture was aliquoted to each well of 6-well plates containing confluent monolayers of Vero cells. The plates then were incubated at 37 °C for 1 h with gentle shaking every 15 min. After 1 h, both carrageenan and unadsorbed viruses were removed, subjected to PBS wash and overlaid with 2 ml of 1% agarose (SeaPlaque, FMC, USA) in RPMI-1640 containing 2% FBS in sequence. The plates then were incubated for 4 days at 37 °C. At due time, the cells were fixed with formalin, stained with 1% crystal violet, and counted for plaque numbers. The number of plaques was calculated as reciprocals of carrageenan at different concentrations in comparison to that of the control (virus with no carrageenan).

2.4. Influence of time of treatment on anti-EV 71 activity

2.4.1. Plaque reduction rate

The inhibitory effect of carrageenan on virus infection was determined by the plaque reduction assay. For pre-treatment group: 1 mg of carrageenan in 1 ml RPMI-1640 medium was added to each well and incubated at 37 °C for 1 h. EV 71 (50 PFU/well) then was added to each well. The plates were incubated at 37 °C for 1 h with gentle shaking every 15 min. After incubation, both carrageenan and unadsorbed virus were removed. The cells were subjected to PBS wash, overlaid with 2 ml of 1% agarose (SeaPlaque, FMC, USA) in RPMI-1640 containing 2% FBS, and incubated at 37 °C for 4 days in sequence. At due course, the cells were fixed with formalin and stained with 1% crystal violet. Plaque numbers then were counted. The number of plaques was calculated as reciprocals of carrageenan at different concentrations in comparison to that of the control (virus with no carrageenan). For post-infection group: EV 71 (50 PFU/well) was suspended in 1 ml RPMI-1640 medium, added to each well and incubated at 37 °C for 1 h. One milligram carrageenan in 1 ml RPMI-1640 medium was added to each well and incubated at 37 °C for another 1 h. The following steps were the same as those in the pre-treatment experiment. All experiments were performed three times and each in triplicate. For adsorption group: the inhibitory effect of carrageenan on virus infection was determined by the plaque reduction assay. One milligram carrageenan in 1 ml RPMI-1640 medium was mixed with an equal volume of infectious EV 71 (50 PFU/well) in RPMI-1640 medium. The carrageenan and EV 71 were co-incubated at 4 °C for 1 h, and then aliquoted to each well of 6-well plates containing confluent monolayers of Vero cells. The plates were incubated at 37 °C for 1 h. The remaining steps were the same as described above. All experiments were performed three times and each in triplicate (Shih et al., 2003; Talarico and Damonte, 2007).

2.4.2. Viral RNA expression level

The RNA expression of Vero cells infected with EV 71 was determined by two methods: real-time RT-PCR and RT-PCR analyses. The antiviral activity was evaluated on the basis of the expression level

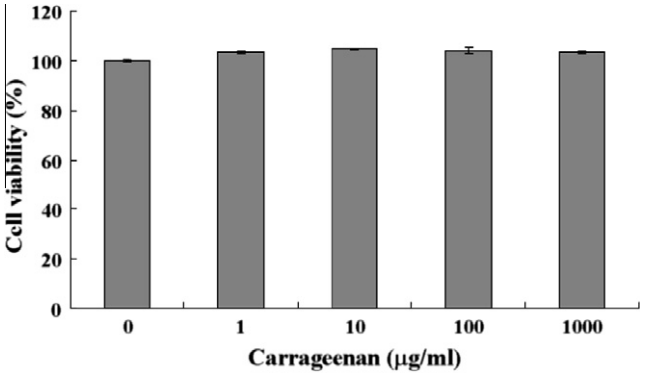

Fig. 1. Cytotoxicity of carrageenan. Vero cells were treated with carrageenan. The cell viability was determined by the MTS method. The percentage of viability is expressed as a comparison to that of cells alone (without carrageenan). Each value was expressed as mean ± SEM from triplicate independent experiments. The asterisks indicates significant differences of $p < 0.05$ (*).

of viral RNA. The antiviral activity of carrageenan (1 mg/ml) for the prophylaxis experiment was performed: Vero cells were pre-treated with carrageenan for 1 h. EV 71 (m.o.i = 0.1) suspended in 1 ml RPMI-1640 medium was added to each well that then was incubated at 37 °C for 1 h. After incubation, both carrageenan and unadsorbed viruses were removed. The cells were subjected to PBS wash. The cells were added with 1 ml RPMI-1640 medium + 2% FBS and then incubated at 37 °C for 48 h. The antiviral activity of carrageenan for the treatment experiment was determined. EV 71 (m.o.i = 0.1) was suspended in 1 ml RPMI-1640 medium and added to each well. The plates were incubated at 37 °C for 1 h. Then, 1 mg of carrageenan in 1 ml RPMI-1640 medium was added to each well and incubated at 37 °C for 1 h. After incubation, both carrageenan and unadsorbed viruses were removed. The cells were subjected to PBS wash and added with 1 ml RPMI-1640 medium + 2% FBS. The treated cells then were incubated at 37 °C for 48 h. At due time, total RNA was extracted from cells using QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany) and RNase Mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instruction. For quantification of RNA, an iCycler IQ (Bio-Rad) employing SYBR® Green technology was used. The primers used were listed: EV 71 (forward primer, 5′-CCTGAAT GCGGCTAATCC-3′, reverse primer, 5′-CCCAAAG TAGTCGGTTCC-3′); GAPDH (forward primer, 5′-GCGACTTCAA CAGCAACTC-3′, reverse primer, 5′-GGTCCAGGGTTT CTTACTCC-3′). Each 25 μl reaction mix contained 5 μl of RNA sample in 1 × RT-PCR buffer (with final concentrations of 10 mM Tris–HCl pH 8.4, 50 mM KCl, 0.01% w/v gelatin and 10 mM DTT, 2.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates, 100 nM of each primer and 100 units M-MLV RT) (Promega, Madison, USA). Reverse transcription was allowed to proceed for 1 h at 37 °C, and then 2 units of Taq DNA polymerase (Invitrogen, California, USA) were added to each reaction tube. PCR amplification and detection were performed using the following conditions: 95 °C for 3 min (1 cycle), 40 cycles of 95 °C for 30 s and 58 °C for 1 min. For quantification of RNA, an iCycler IQ (Bio-Rad) was used. The primers were listed as follows: EV 71 VP1 (forward primer, 5′-GTGGC AGATGTGATTGAG-3′, reverse primer, 5′-GTTAT GTCTATGTCCCAGT T-3′); GAPDH (forward primer, 5′-ACCACAGTCC ATGCCATCAC-3′, reverse primer, 5′-GGTCCAGGGTTTCTTACTCC-3′). The remaining steps were the same as stated above. All determinations were performed three times and each in triplicate.

2.5. The binding activity of carrageenan to EV 71 detected by ELISA

Carrageenan was covalently immobilized on a universal binding plate (Corning Inc., NY, USA). Each well was treated with a solution of 10 mM acetate buffer (pH 4.0) at 25 °C for 1 h, irradiated under UV light at A_{254} for 1 min, blocked with 100 μL solution of PBS + 2% BSA at 25 °C for 1 h, and washed with PBS in sequence. EV 71 ($\sim 10^5$ PFU/well) then was added to the carrageenan-immobilized wells and incubated at 4 °C for 12 h. The virons that bind to carrageenan were reacted with anti-EV 71 antibodies. The bound antibodies were subjected to detection using the staining method of horseradish peroxidase-conjugated goat anti-mouse IgG Fc (1:1000; Chemicon, Temecula, CA) and *o*-phenylenediamine dihydrochloride (OPD) (Sigma Chemical Co.). Absorbance readouts were recorded at A_{490} using an ELISA reader (Hidari et al., 2008; Totani et al., 2003).

2.6. Electron microscope

To determine whether carrageenan can directly adsorb virus particles, the examination of transmission electron microscopy (TEM) was performed. For control: EV 71 alone was examined. For the carraageenan-EV 71 complex: carrageenan was mixed with EV 71 and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 60 min. The pellets were stained en bloc with uranyl acetate, post-fixed with 1% osmium tetroxide in 0.1 phosphate buffer (pH 7.2) for 1 h, dehydrated in ethanol and propylene oxide, and embedded in Epon/Araldite in sequence. Ultrathin sections were collected on copper grids and stained with uranyl acetate

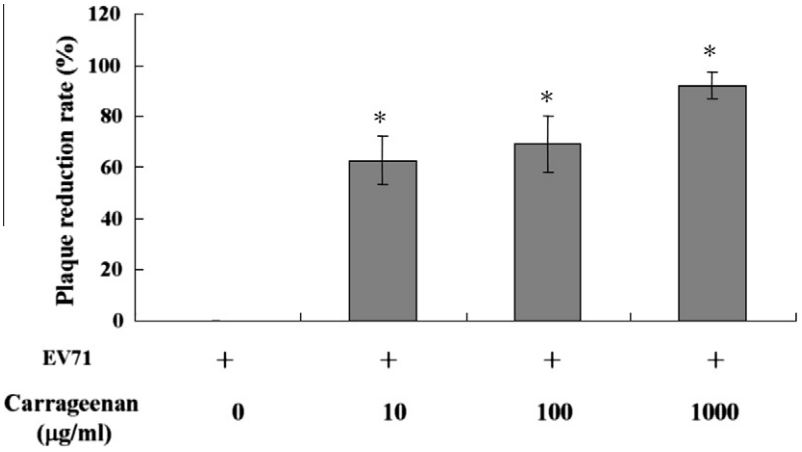

Fig. 2. Effect of carrageenan on virus infection. Vero cells were pre-treated with (1000, 100, 10 μg/ml of carrageenan) or without carrageenan for 1 h and then infected with EV 71 (50 PFU/well) (please see the 'Section 2' for detailed experiment procedures). Each value was expressed as mean ± SEM from triplicate independent experiments. The asterisks indicates significant differences of $p < 0.05$ (*).

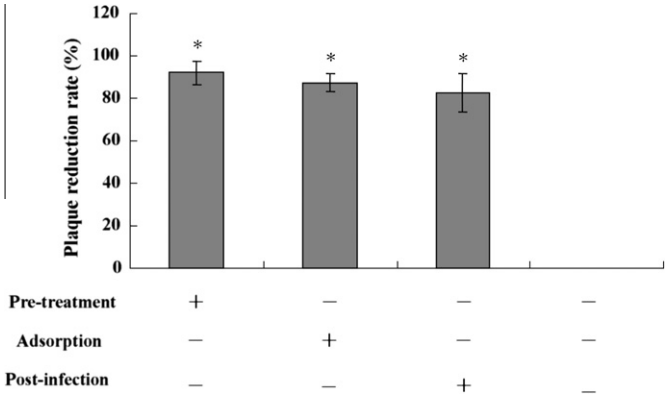

Fig. 3. Influence of time of treatment with carrageenan on EV 71 infection. Vero cells were pre-treated with carrageenan (1 mg/ml) and then infected with EV 71 (50 PFU/ml) as a pre-treatment group or carrageenan was added to Vero cells simultaneously with viruses as an adsorption group. Vero cells pre-infected by EV 71 (50 PFU/ml) and then treated with carrageenan serve as post-infection group (please see the 'Section 2' for detailed experiment procedures). The anti-EV71 effect of carrageenan was determined by the plaque assay, whereby plaque reduction rates were calculated. Each value was expressed as mean ± SEM from triplicate independent experiments. The asterisks indicates significant differences of $p < 0.05$ (*).

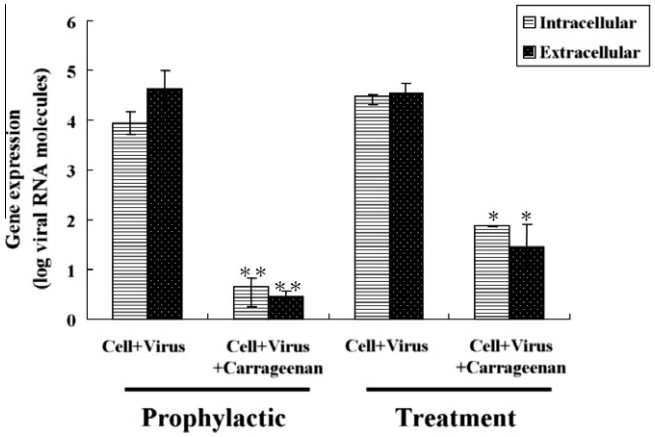

Fig. 4. Inhibition of EV 71 gene expression by carrageenan. The prophylactic group: Vero cells were pre-treated with carrageenan and then infected with EV 71 (m.o.i = 0.1). The treatment group: Vero cells were pre-infected by EV 71 (m.o.i = 0.1) and then treated with carrageenan. The total RNAs were extracted from cells at 48 h post-infection. The RNA expressions of the EV 71 infected Vero cells were evaluated by real-time PCR. Each value was expressed as mean ± SEM from triplicate independent experiments. The asterisks indicates significant differences of $p < 0.05$ (*).

and Sato's lead for the TEM examination. The section images were snapshotted using a Hamamatsu AMT Advantage HR CCD camera (Hamamatsu Photonics; Hamamatsu City, Shizouka Pref, Japan) equipped on a Hitachi 7500 electron microscope (Hitachi High Technologies Corporation; Tokyo, Japan) in operation at 80 kV.

2.7. The apoptosis of EV 71 infected Vero cells

For the apoptosis assay, carrageenan (1 mg/ml) was added in cells before virus infection. The carrageenan-added cells was incubated at 37 °C for 1 h, and added with a suspension of EV 71 (m.o.i = 0.1; in 1 ml RPMI-1640 medium). The cell mixture then was incubated at 37 °C for another 1 h. After incubation, both carrageenan and unadsorbed virus were removed. The cells were subjected to PBS wash, addition of 1 ml RPMI-1640 medium + 2% FBS and incubation at 37 °C for 1 h in sequence. The cells then were stained with Annexin-V-fluorescein and propidium iodide (Roche) following the manufacturer's instruction and subjected to flow cytometry analysis (FACScan, Becton Dickinson, Mountain View, CA) (Lin et al., 2009).

2.8. Statistical analysis

The graphs and statistical analyses were performed using SigmaPlot® and SigmaStat®. The statistical analyses between groups of test animals were determined by one way Anova and

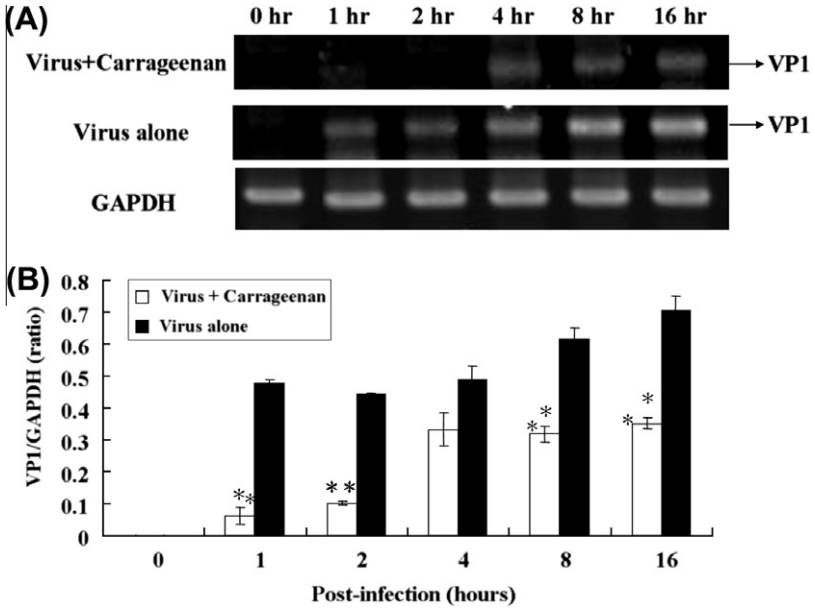

Fig. 5. Influence of treatment time on viral RNA synthesis by carrageenan. Carrageenan was added at 0 h and the EV 71 VP1 mRNA expressions were detected at 0, 1, 2, 4, 8, 16 h post-infection. The reductions of VP1 mRNAs expressions are proportional to the time of carrageenan added. (A) The RNA expressions in EV71 infected Vero cells were evaluated by RT-PCR. The RT-PCR products were examined by electrophoresis on 1% agarose gels loaded with 5 μl in each well. (B) The levels of VP1 mRNA expressions were quantified using densitometer. Each value was expressed as mean ± SEM from triplicate independent experiments. The asterisks indicates significant differences of $p < 0.05$ (*).

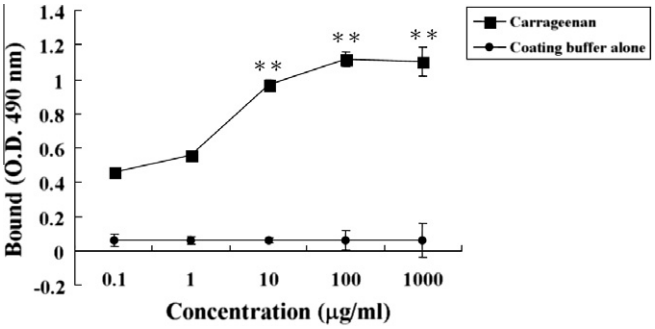

Fig. 6. Binding effect of carrageenan to EV 71. Carrageenan was covalently immobilized on a plate. The EV 71 virus was added to the carrageenan-immobilized wells. The virons that bind to carrageenan were reacted with the anti-EV 71 antibody and detected using the Goat anti mouse IgG-Fc HRP. The data are the means ± SD of triplicate experiments. $^{**}p < 0.01$ was considered statistically significant compared with low concentration.

Tukey HSD test. Differences were considered significant if the *P* value was <0.05.

3. Results

3.1. Inhibition of EV 71 infection by carrageenan

Cell viability assays were performed to determine whether carrageenan has the anti-EV 71 activity. Before doing so, we first assessed cytotoxicity of carrageenan as they may possess unforeseen toxicity. Cell viability was counted by the MTS method, whereby carrageenan was concluded carrying no cytotoxicity to the cells tested (Fig. 1). Next, plaque reduction assays were performed to see if carrageenan can interfere with the virus-cell association. As shown in Fig. 2, the reduction of plaque numbers is proportional to the doses of addition of carrageenan.

3.2. Influence of time of treatment with carrageenan on EV 71 infectivity

The mode of action of carrageenan effective against the EV 71 infection was studied. Addition of carrageenan at a dose of 1 mg/ml was set out as the effective dose in inhibitory assays. A time course experiment was performed to observe the influence of addition of carrageenan during virus multiplication cycles. First, Vero cells were pre-treated with carrageenan for 1 h and then infected with EV 71 as a pre-treatment group. Second, Vero cells were treated with carrageenan simultaneously with virus as an adsorption group. Third, Vero cells were treated with carrageenan at 1 h after adsorption of virus to the cells as a post-infection group. A high inhibitory effect was observed in the group pre-treated with carrageenan (92%), which is better than the group simultaneously treated with the virus (87%) or the post-infection group (82%) (Fig. 3).

3.3. Carrageenan inhibits viral gene expression of EV 71 infected Vero cells

Soon after viral entry into host cells the synthesis of viral RNAs normally takes place in a normal EV 71 replication cycle. Of this consideration, the total RNAs were extracted from cells at 48 h post-infection and subjected to quantitative PCR and RT-PCR assays. As shown in Fig. 4, the levels of the EV 71 mRNA were found significantly decreased in the EV 71-infected Vero cells pre-treated with carrageenan. The expressions of the EV 71 VP1 mRNA were measured at 0, 1, 2, 4, 8, 16 h post-infection. As shown in Fig. 5, the expression of the VP1 mRNA in the group receiving carrageenan is significantly lower than that receiving no carrageenan. As a result, carrageenan is concluded able to suppress virus mRNA synthesis in the EV 71-infected cells.

3.4. Carrageenan adsorbs EV 71 particles

Many untreated algal sulfated polysaccharides have previously been found with activities against viral infections, which may be regarded as an ensemble effect in associating viruses. To explore whether carrageenan can directly interact with EV 71, carrageenan was first immobilized on a universal binding plate and added with a solution containing viruses. Carrageenan apparently can bind with EV 71 (Fig. 6) in a dose-dependent manner. To further determine whether carrageenan can directly adsorb virus particles, the interaction was visualized through transmission electron microscopy (TEM). First carrageenan (1 mg/ml) was co-incubated with EV 71 (10^7 PFU/ml); the mixture then was examined under TEM. The viral particles displayed in spherical morphology, ~30 nm in diameter (Fig. 7A). Carrageenan and EV 71 were found to form complexes, which had a size about 100 nm (Fig. 7B). The formation of carrageenan-EV 71 complexes suggests carrageenan adsorbs EV 71 particles so as to prevent viruses from entering into cells.

3.5. Inhibition of EV 71-induced apoptosis

Flow cytometry experiments were performed to interrogate the mechanism whereby the EV 71-infected cells were prevented from death. In the flow cytometry assays, Annexin-V-fluorescein and propidium iodide were used to stain the EV 71-infected cells. The

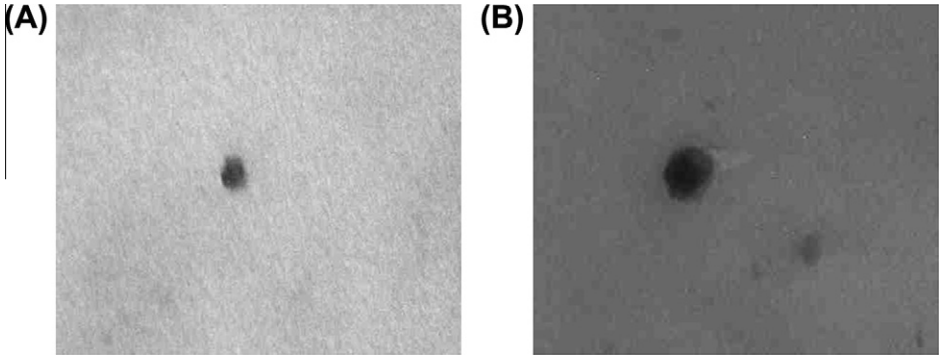

Fig. 7. Electron microscopy images of EV 71 (A) and EV-carrageenan complexes (B). The particle size of the carraageenan-EV 71 complex is larger than that of EV 71. The diameters of the EV 71 particle and the carraageenan-EV 71 complex were estimated to be 30 nm and 100 nm, respectively. (Magnification, 60,000×; bar, 100 nm).

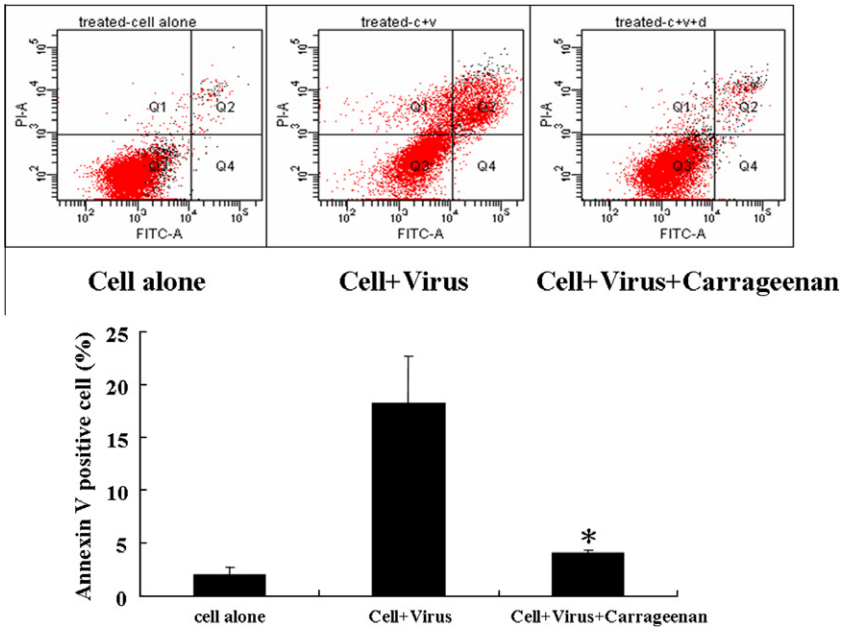

Fig. 8. Inhibition effects of carrageenan on EV 71-induced apoptosis. In the apoptotic assay, carrageenan was added in Vero cells prior to virus infection. The cells were stained with Annexin-V-fluorescein and propidium iodide and measured using flow cytometry. Each value was expressed as mean ± SEM from triplicate independent experiments. The asterisks indicates significant differences of $p < 0.05$ (*).

fluorescence drifting stands for cells undergoing apoptosis. With addition of EV 71, Vero cells showed a significant fluorescence drift to the upper right quadrant (Q2) in comparison to the control (Fig. 8). While, with inclusion of carrageenan this fluorescence drifting decreased, corresponding to reduction of EV 71-induced apoptosis from 18.2% to 4.05%. This result suggests that carrageenan inhibits the EV 71-induced apoptosis in Vero cells.

4. Discussion

Carrageenan exists widely in red algae (Rhodophyta) (Campo et al., 2009). Though previous studies have shown that carrageenan possesses varied anti-viral activities effective against such viruses as influenza A (H1N1) virus (Leibbrandt et al., 2010; Wang et al., 2011), dengue virus-2 (DENV-2) (Talarico et al., 2011; Talarico and Damonte, 2007), herpes simplex virus (HSV) (Carlucci et al., 1999; Mateu et al., 2011), human rhinoviruses (HRV) (Grassauer et al., 2008), hepatitis A virus (HAV), and human papillomaviruses (HPV) (Roberts et al., 2007), the capability of carrageenan inhibiting EV 71 infection however was not verified.

The antiviral activity of sulfated polysaccharides was proposed to effect at the early stage of virus infection by virtue of interfering virus adsorption and internalization (Duarte et al., 2001; Matsuhiro et al., 2005; Mazumder et al., 2002; Talarico et al., 2004). More specifically speaking, carrageenan may bind to HS residues of cellular proteoglycans (Damonte et al., 2004; Talarico and Damonte, 2007). Our result revealed that carrageenan preventing EV 71 from infections may follow the same mechanism, that is, disrupting virus-receptor binding and the subsequent internalization of nucleocapsid into cytoplasm (Fig. 2, Fig. 3). Though virus-carrageenan complexes may still be able to enter into cells, virions probably were not released from endosomes as suggested by the infectious center and virion uncoating assays. The expression of EV 71 mRNAs was significantly reduced upon addition of carrageenan in the EV 71-infection cells (Fig. 4, Fig. 5).

Lactoferrin has been known able to prevent certain viruses from entering into host cells likely by blocking viral receptors, and/or directly binding to virus particles (van der Strate et al., 2001) through hydrophobic interactions between viruses and polysaccharides. The sulfated seaweed polysaccharides that affect the antiviral activity may follow the same mechanism as lactoferrin by forming polysaccharide-virus complexes; thereby, virus is no longer able to interact with receptors. A minimal structure with ionic and hydrophobic moieties in seaweed polysaccharides has been put forward as the effective region in a fashion similar to the minimal binding structure in HS/heparin (Damonte et al., 2004). The EV 71 viral particle has also been shown having a specific and significant binding affinity to native fucoidan immobilized on a plastic plate in a dose-dependent manner, suggesting that sulfated seaweed polysaccharides can bind to viruses in addition to the fact that the diameters of viral particles become enlarged when carrageenan was added in (~30 nm vs. 100 nm; Fig. 7).

On the basis of previous reports (Duarte et al., 2001; Matsuhiro et al., 2005; Mazumder et al., 2002; Talarico et al., 2004), the antiviral activity of sulfated polysaccharides should effect in the early stage of virus infection by interfering with virus adsorption and internalization. Carrageenan successfully ameliorated symptoms of common cold and viral load in a recent clinical trial (Eccles et al., 2010), favoring carrageenan a suitable antiviral compound in prevention of EV71 infections.

Natural products particularly from ethnobotanical sources have been extensively screened for new and more effective EV 71 inhibitors. For example, crude extracts from *Salvia miltiorrhiza* (danshen) and *Ocimum basilicum* were able to inhibit EV 71-induced CPE in cell lines (Chiang et al., 2005; Wu et al., 2007). Allophycocyanin (104 kDa) from cyanobacterium *Spirulina platensis* was capable of inhibiting EV 71 (Shih et al., 2003). The extracts from *Houttuynia cordata* were able to suppress the apoptotic process in EV 71-infected Vero cells by inhibiting viral replication (Lin et al., 2009). Nonetheless, carrageenan still has an edge over others, thanks to carrying such advantages as low production cost, broad spectrum of antiviral activities, low toxicity, high safety, wider acceptability, and novel mode of action.

In conclusion, our results indicated that carrageenan is a superb inhibitory agent effective against EV 71 infection. The mechanism of the inhibition is likely through forming virus-polysaccharide complexes to prevent viruses from entry into cells. At the current

stage, carrageenan may be suitable serving as a health food supplement for daily antiviral prevention.

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