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Original Article

Role of Rotavirus Enterotoxin NSP4 in the Inflammatory Response in Murine Macrophage RAW 264.7 Cells

Song ah Kim, Van Thai Than and Wonyong Kim*

Department of Microbiology, Chung-Ang University College of Medicine, Seoul, Korea

The rotavirus nonstructural glycoprotein, NSP4, has been identified as the first viral enterotoxin capable of inducing diarrhea. To investigate the biological function of NSP4 in the inflammatory process, a cDNA from human rotavirus (Wa strain) RNA segment 10 was amplified by RT-PCR, cloned into TA vector, and subsequently subcloned into pET23b expression plasmid. The expression of NSP4 protein was determined by SDS-PAGE and Western blotting, then, the protein was purified by affinity chromatography on Ni-NTA-agarose column. The inflammatory effects of NSP4, namely, production of nitric oxide (NO), pro-inflammatory cytokines (IL-1β, IL-6, IL-10, and TNF-α), and prostaglandin E2 (PGE_2), was evaluated using NSP4-stimulated RAW 264.7 murine macrophages and compared with those observed after stimulation with lipopolysaccharide (LPS). The levels of IL-1β, IL-6, and TNF-α were significantly increased, and those of NO and PGE_2 also increased in NSP4-stimulated RAW 264.7 cells. These findings indicate that NSP4 plays an important role in the inflammatory response observed during rotavirus infection.

Key Words: Rotavirus, NSP4, RAW 264.7 murine macrophage, Inflammation

INTRODUCTION

Rotavirus is the predominant etiological agent causing acute viral gastroenteritis in the young ones of a wide range of mammalian and avian species, including human infants (1). About 611,000 children die every year because of rotavirus infection, and over two million are hospitalized (2). Recently, extrahepatic biliary obstruction and biliary atresia have been associated with rotavirus infection. This indicates the increasing importance of controlling the spread of this organism (3).

Rotaviruses are members of the *Reoviridae* family and contain 11 double-stranded RNA segments (1). The rotavirus gene 10 segment encoding the nonstructural glycoprotein 4 (NSP4), and a peptide corresponding to residues 114~135 has been suggested to function as a viral enterotoxin and play a role in the pathophysiological mechanism whereby rotaviruses induce diarrhea (4). NSP4 may function by causing Ca^{2+} influx into the cytoplasm of infected cells (5, 6). To study the biological properties of NSP4, the expression of large quantities of purified protein was studied in various eukaryotic and prokaryotic gene expression systems, including *Escherichia coli* (7, 8).

Inflammation is inherent to the pathogenesis of many diseases. Macrophages are one of the critical immune cells

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*Corresponding author: Wonyong Kim, PhD. Department of Microbiology, Chung-Ang University College of Medicine, 84 Heukseok-ro, Dongjak-gu, Seoul 06974, Korea.
Phone: +82-2-820-5685, Fax: +82-2-822-5685, e-mail: kimwy@cau.ac.kr
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involved in the regulation of inflammatory response. Activated macrophages secrete different inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), prostaglandin E2 (PGE_2), and nitric oxide (NO) (9).

Cytokines such as TNF-α, IL-1β, IL-6, and IL-10 are soluble proteins secreted by the immune cells; they can alter the properties of different cell types and provide essential communication signals to the motile immune cells (10), but excessive and uncontrolled production of these inflammatory cytokines may lead to serious systemic complications such as microcirculatory dysfunction, tissue damage, and septic shock, which can exact a high mortality (11).

PGs are generated by many cell types, including activated macrophages (12). The rate-limiting enzyme in PG synthesis is cyclooxygenase (COX). COX-2 is induced by several stimuli, including growth factors, mitogens, cytokines, and tumor promoters. Its uncontrolled activity has been hypothesized to be responsible for the pathogenesis of many chronic inflammatory disorders (12). NO is synthesized from L-arginine by nitric oxide synthase (NOS). iNOS is considered as the most important enzyme involved in the regulation of inflammatory response. iNOS-induced overproduction of NO has been implicated in the pathology of several inflammatory disorders, including septic shock, inflammation-induced tissue damage, and rheumatoid arthritis (13). Thus, these inflammatory mediators are critically involved in the pathogenesis of a variety of human inflammatory diseases, including inflammatory bowel disease, multiple sclerosis, and rheumatoid arthritis.

Until recent, the critical roles of the NSP4 in rotavirus pathogenicity have not been clearly understood. In 2007, Borghan and colleagues investigated that the NSP4 expression -induced production of NO by observing the transcriptional changes in the inducible NO after rotavirus infection in mice model and treatment of the mouse macrophage RAW 264.7 cells (14). In 2010, Ge and colleagues identified that the purified-NSP4 of rotavirus-infected Caco-2 cells triggers the secretion of inflammatory cytokines such as TNF-α, IL-6, and IL-8 in macrophage-like THP-1 cells (15). However, these studies did not yield enough information, therefore, more studies are needed to elucidate the function of inflammation-associated NSP4 in rotaviruses.

In this study, the rotavirus enterotoxin NSP4 gene from the human Wa strain was cloned into pET23b expression plasmid containing His-tag sequence, which enabled us to purify the gene product. The protein was subsequently used, for the first time, to identified the correlated influence of the inflammatory effects of NSP4 on the production of NO, pro-inflammatory cytokines (IL-1β, IL-6, IL-10, and TNF-α), and PGE_2 was evaluated using NSP4-stimulated RAW 264.7 murine macrophages. In addition, the effects were compared with those of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages.

MATERIALS AND METHODS

Cell culture and viruses

MA104 cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA) for rotavirus Wa cultivation. Murine macrophage-like RAW 264.7 cells were selected for use in the investigation of the inflammatory effects of NSP4, because macrophages play an important role in both host defense and inflammation. Murine RAW 264.7 macrophage line ATCC TIB-71™ (ATCC, Manassas, VA, USA) was acquired from the Korea Cell Bank (Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and gentamicin (20 μg/ml; GIBCO BRL). The human rotavirus Wa G1P1A[8] strain was purchased from the ATCC. The virus was then propagated onto the MA104 cells and stored at -80℃ for further examination.

RNA extraction

Rotavirus dsRNA was extracted using the Trizol method (Gibco BRL). In brief, 0.3 ml of the culture supernatant was mixed with 0.7 ml of Trizol reagent and 0.2 ml of chloroform:isoamylalcohol mixture (24:1). After centrifugation at 10,000 g for 15 min, the RNA in the aqueous solution was precipitated by adding an equal volume of isopropanol. The RNA precipitate was collected by centrifugation

at 14,000 g for 10 min, washed with 70% ethanol, and finally dissolved in 20 μl of RNase-free water.

Cloning of NSP4 gene

The extracted dsRNA genome of rotavirus Wa strain was subjected to one-step RT-PCR using specific primers for the open reading frame of NSP4 gene amplification. The primers were designed by aligning the sequences of six NSP4 cDNA of human rotaviruses (GenBank accession numbers AF200224, FJ423133, FJ423144, JQ713651, HM627562, and JQ043303) from NCBI GenBank by using Primer 3 software with default settings (http://primer3.sourceforge.net/). The primers used to amplify the open reading frame of NSP4 were NSP4-NdeIF (5'-ATTCCATATGGACAAAC-TGGCGGACCTGAACTACACATTGAGTGCA-3') and NSP4-XhoIR (5'-ATTCCTCGAGCATGGATGCAGTC-ACTTC-3'). PCR amplification was performed using 30 cycles, with each cycle consisting of a denaturation step at 95℃ for 1 min, a primer annealing step at 56℃ for 1 min, a primer extension step at 72℃ for 1 min, and one cycle at 72℃ for 10 min. The amplified PCR product was inserted into a TA cloning vector (RBC, T&A Cloning kit, Taipei, Taiwan), transformed into *E. coli* DH5α (RBC, HIT Competent Cells™, Taipei, Taiwan) and sequenced using a Big-Dye terminator cycle sequencing kit and an automatic DNA sequencer (Model 3730; Applied Biosystems, Foster City, CA, USA). M13 promoter and M13-pUC reverse universal primers were used for sequence analysis.

Expression of NSP4 protein

NSP4 cDNA was excised from the recombinant plasmid TA cloning vector using NdeI and XhoI restriction enzymes (New England Biolabs, Beverly, MA, USA) and cloned into the expression plasmid pET23b (Novagen, Madison, WI, USA), previously digested with NdeI and XhoI. The recombinant plasmid was verified by PCR and restriction enzyme digestion. The pET23b plasmid containing NSP4 cDNA was transformed into *E. coli* BL21 (DE3) strain. Overnight cultures of the transformants grown in Luria-Bertani medium were diluted, grown to attain an optical density (OD_{550}) of 0.5~0.7 and induced with isopropylthio-α-D-galactoside (IPTG) to a final concentration of 1 mM and were incubated for an additional 4 h. Whole cell lysates, the soluble and insoluble fractions, obtained following sonication and centrifugation, were prepared and solubilized in lysis buffer (0.5 M KCl, 0.02 M Tris-HCl, 0.01 mM), mixed with sample buffer, separated by electrophoresis (12% SDS-PAGE), and stained with Coomassie brilliant blue G-250. For western blotting, the electrophoresed proteins were electroblotted onto nitrocellulose membrane in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 1.0A for 30 min. NSP4 was visualized using His-tag polyclonal antiserum and an anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). The membrane was exposed to Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK) for visualization.

Purification of His-tagged NSP4 from *E. coli*

Purification of 6×His-tagged NSP4 protein by Ni-NTA affinity chromatography was performed under denaturing conditions. The harvested cells were resuspended in 20 ml of lysis buffer (500 mM KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$, and 4 mg/ml lysozyme). The cells were incubated on ice for 30 min and then sonicated on ice. The suspension was sonicated for 40 s pulses, at least 1 min long, at 2 s intervals, by using a microtip (Branson Ultrasonic Co., Danbury, CT, USA). The recombinant protein in the form of inclusion bodies was separated from the soluble lysate by centrifugation at 12,000 × *g* for 15 min. The precipitate was resuspended in 20 ml of 8 M urea lysis buffer (8 M urea, 500 mM KCl, 20 mM Tris-HCl). The mixture was centrifuged at 12,000 × *g* for 15 min and the supernatant was loaded on a column (10 ml Poly-Prep® Chromatography Columns; Bio-Rad, Hercules, CA, USA) with 4 ml of His-Bind agarose resin precharged with Ni ion (75% slurry; Elpis, Daejeon, Korea) previously equilibrated with 10 ml of 8 M urea lysis buffer. To remove unbound protein, the column was washed with 20 ml of 8 M urea buffer (containing 5~60 mM imidazole). Derivatized protein was eluted with 8 M urea lysis buffer plus 300 mM imidazole. The protein solution was stored at -20℃. All pellets and supernatants were analyzed

by SDS-PAGE. After adjusting the concentration of recombinant NSP4 protein, bacterial endotoxin levels at each purification step were assessed using the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Waltham, MA, USA).

MTT assay for cell viability

RAW 264.7 murine macrophages were cultured in DMEM containing 10% heat-inactivated FBS and 20 μg/ml gentamicin at 37℃ in a water-jacketed CO_2 incubator (Model 3111; Thermo Fisher Scientific). Cell viability was determined with 1, 5, 10, 50, 100, 500, and 1,000 pM various NSP4 proteins by using TC10™ automated cell counter (BioRad). RAW 264.7 cells were plated at a density of 5×10^5 cells/well in 24-well plates (Nunc, Roskilde, Denmark). After overnight incubation, the cells were treated with various concentrations of NSP4 in 1 ml of serum-free medium. After 24 h treatment, the cells were subjected to 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The medium was replaced by 500 μl of fresh serum-free medium containing 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA). After 30 min incubation at 37℃ in an atmosphere containing 5% CO_2 and 95% air, the MTT-containing medium was removed, and the reduced formazan dye was solubilized by adding 500 μl of DMSO into each well. After gentle mixing, the absorbance was monitored at 590 nm by using a plate reader (Infinite® F200; Tecan, Männedorf, Switzerland).

Nitrite determination

RAW 264.7 cells were plated at a density of 5×10^5 cells in 24-well plates with 1 ml of the culture medium and incubated for 24 h. The cells were treated with 1, 5, 10, 50, 100, and 500 pM NSP4 protein and incubated for additional 24 h. The LPS from *E. coli* 0127:B8 (Sigma-Aldrich) was used as a positive control. NO content in the culture medium was determined using the Griess reaction, because the stable NO-oxidation product, namely, nitrite produces a chromophore with the Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2% phosphoric acid). Briefly, 100 μl of additional the cell culture medium was added to each well of a 96-well plate. After the addition of 100 μl of the Griess reagent, the plate was left for 5 min at room temperature under shaker conditions. The absorbance was measured at 540 nm by using a spectrophotometer and the nitrite concentration was calculated by comparing the concentration with standard solutions of sodium nitrite.

Measurement of IL-1β, IL-6, IL-10, PGE_2, and TNF-α by ELISA

The levels of IL-6, IL-10, IL-1β, PGE_2, and TNF-α in the supernatant from macrophage culture was determined using an ELISA kit, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). RAW 264.7 cells were pretreated with FBS-free medium for 3 h for activation and then stimulated with NSP4 (in a concentration-dependent manner) for 24 h. The supernatant was collected and stored at -80℃ until analysis.

RESULTS

Cloning and expression of NSP4

The NSP4 (Wa strain, G1P1A[8]) recombinant gene cassette were confirmed by PCR, which produced approximately 750 bp sized amplicon (data not shown). DNA sequencing confirmed that the NSP4 recombinant gene cassette was fused in-frame into the plasmid pET23b due to the artificially incorporated restriction sites. As expected, the experimental expression of the NSP4 recombinant protein demonstrated approximately 20 kDa protein in size on 10% SDS PAGE (Fig. 2A) and western blot analyses (Fig. 2B). In addition, the experimental expression demonstrated that the peak expression level of the NSP4 recombinant protein is at 4 h of incubation at 37℃ in the present of 1 mM IPTG (Fig. 1).

Purification of His-tagged NSP4 by one-step Ni^{2+} affinity chromatography

E. coli pET23b transformants were grown to the late exponential phase and the expression of the cloned gene was induced by 1 mM IPTG. Cells were harvested after 4 h of

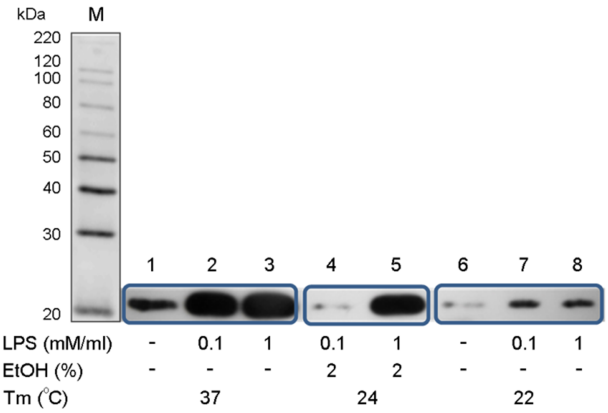

Figure 1. Expression of recombinant NSP4. Recombinant NSP4 was expressed by induction with IPTG, as shown by western blotting: lane 1~3: 0, 0.1, and 1 mM IPTG at 37℃, respectively; lane 4~5: 0 and 1 mM IPTG at 37℃, 2% EtOH; and lane 6~8: 0, 0.1, and 1 mM IPTG at 22℃, respectively.

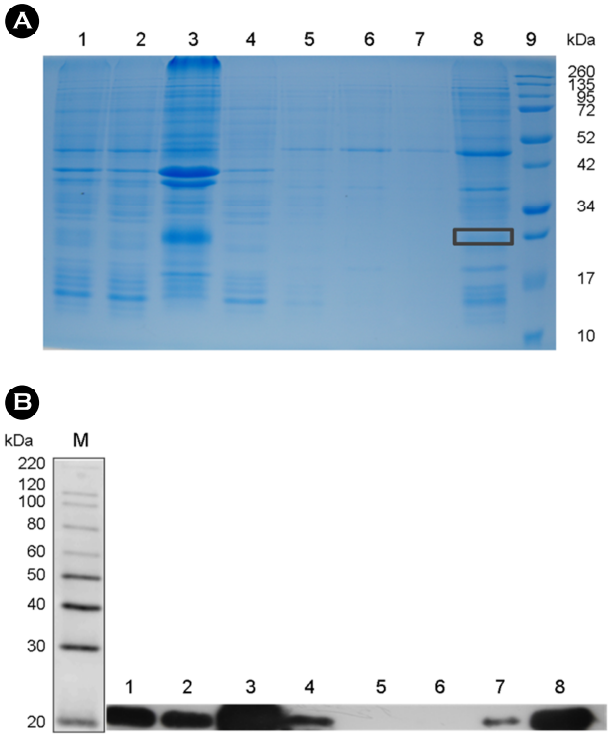

Figure 2. Purification of recombinant NSP4 after the suspension and dissolution of inclusion bodies. (A) 12% SDS-PAGE of the fraction obtained by Ni-ion affinity chromatography; lane 1: total denatured lysates; lane 2, denatured lysis supernatant; lane 3, precipitate; lane 4, flow-through; lane 5~6, sequential column washings; lane 7, elution of the recombinant NSP4 protein from the column; lane 8, concentration of the recombinant NSP4 protein; and lane 9, protein molecular weight marker (Invitrogen, USA). (B) Western blotting using the monoclonal anti-His antibody of each fraction obtained during purification. Lane 1~8, as in SDS PAGE (panel A).

incubation at 37℃. The washed cells were lysed and the cleared lysate was incubated with Ni-NTA resin in 8 M urea (exposed to denaturation). Loosely bound proteins and remained LPS were washed from the resin by using a wash buffer containing 5~60 mM imidazole. The recombinant NSP4 was eluted with an elution buffer containing 300 mM imidazole and detected by Coomassie brilliant blue staining of the SDS-PAGE gel. PAGE showed that the recombinant NSP4 was reasonably pure for use in experimental studies (Fig. 2A). The protein product of gene 10 is a 20-kDa protein, which becomes 28 kDa after glycosylation. SDS-PAGE analysis of the induced cells containing pET23b produced a ~20-kDa band, when compared to the uninduced cells containing pET23b. For western blotting, the resolved proteins were electroblotted onto a nitrocellulose membrane and reacted with mouse polyclonal antiserum against His-tag (Fig. 2B). LPS LAL assays revealed that the endotoxins were effectively eliminated from recombinant NSP4 proteins at >99% level. This result suggests that Ni^{2+} affinity chromatography effectively reduced endotoxin level of crude whole cell lysate.

Cell viability

MTT assay was used to determine the possible cytotoxic effects of the NSP4 recombinant protein treatment onto the RAW 264.7 macrophage cells. The NSP4 recombinant protein treatment between the doses 1 and 500 pM had no effects on the viability of the RAW 264.7 macrophage cells. In contract, the NSP4 recombinant protein treatment with the doses higher than 1 nM resulted in decrease the viability of the RAW 264.7 macrophage cells. The cell viability of the un-treated NSP4 recombinant protein was used as control and displayed at >98% viability (Fig. 3).

Generation of pro-inflammatory NO and PGE_2 in RAW 264.7 cells

The effects of NSP4 included production of nitrite, a stable

metabolite of NO in RAW 264.7 cells. As shown in Fig. 4A, NO concentration increased in RAW 264.7 cells (in a concentration-dependent manner) when treated with 1, 5, 10, 50, 100, and 500 pM NSP4; the concentration was 3.2 ± 0.5 μM, 31.2 ± 2.8 μM, 33.2 ± 2.1 μM, 36 ± 1.8 μM, 36.2 ± 1.9 μM, and 33.8 ± 1.9 μM, respectively. Consistent with the observations in case of nitrite accumulation, NSP4 caused an increase in PGE_2 production in murine macrophages (Fig. 4B). PGE_2 concentration in the RAW 264.7 cells exposed to 1, 5, 10, 50, 100, and 500 pM NSP4 was 725.0 pg/ml, 9,672.1 ± 5,258.6 pg/ml, 9,325.2 ± 434.9 pg/ml, 23,725.6 ± 713.8 pg/ml, 21,812.0 ± 1,710.8 pg/ml, and 12,026.1 ± 3,859.1 pg/ml, respectively. These results showed that NSP4 induces the production of inflammatory mediators, including NO and PGE_2.

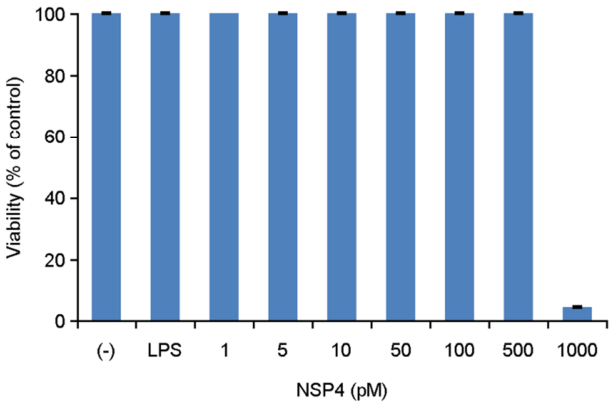

Figure 3. Viability of RAW 264.7 cells after 24-h induction with NSP4. Viability was determined using the colorimetric MTT assay.

Production of cytokines in NSP4-stimulated RAW 264.7 cells

NSP4 protein tested in this study induced increase in IL-1β, IL-6, IL-10, and TNF-α production in RAW 264.7 cells. As is shown in Fig. 5, IL-1β, IL-6, and IL-10 production increased dramatically because of NSP4 treatment. NSP4 was inducing IL-1β, IL-6, IL-10, and TNF-α production up to 50 pM from 500 pM, respectively. NSP4 caused an increase of ~500 pM in IL-1β concentration, while the concentration of IL-10 and TNF-α increased and then decreased (500 to 100 pM and 500 to 5 pM, respectively). The concentration of IL-1β in LPS-stimulated RAW 264.7 cells exposed to 1, 5, 10, 50, 100, and 500 pM NSP4 was -27 pg/ml, -27.8 ± 2.18 pg/ml, 72.1 ± 54.18 pg/ml, 173.5 ± 25.94 pg/ml, 176.9 ± 5.96 pg/ml, 208.8 ± 15.95 pg/ml, and 647.1 ± 38.08 pg/ml, respectively. IL-6 concentration in cells exposed to 1, 5, 10, 50, 100, and 500 pM NSP4s was -3.1 ± 0.07 pg/ml, 301.7 ± 248.32 pg/ml, 544.2 ± 125.73 pg/ml, 1296.8 ± 27.45 pg/ml, 1447.9 ± 31.82 pg/

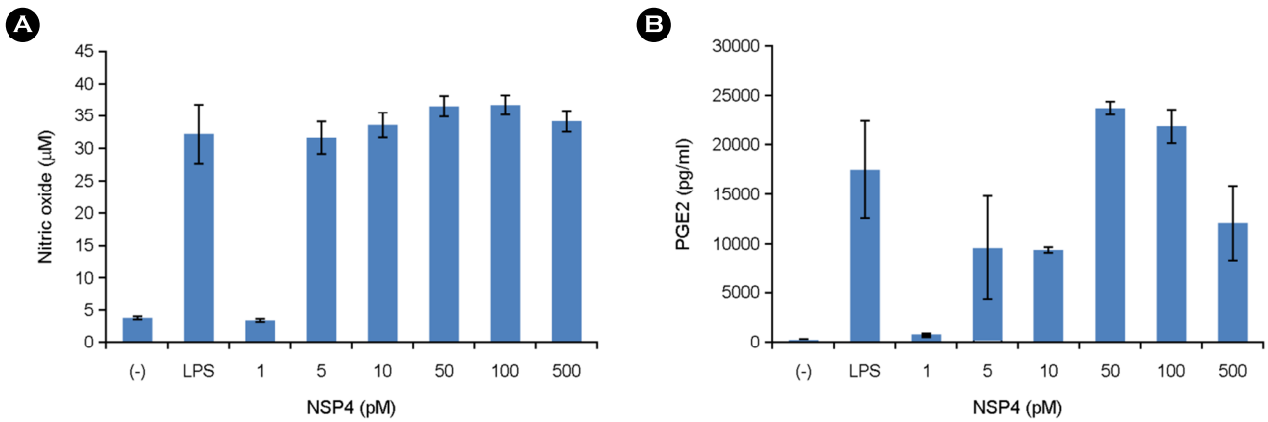

Figure 4. Effects of NSP4 on the levels of NO and PGE_2 in RAW 264.7 cells. Cells were treated for 24 h with 1, 5, 10, 50, 100, and 500 pM of various NSP4s. The concentrations of nitrite and PGE_2 were measured as described in the Materials and Methods. The results are expressed as the mean ± standard error from three independent experiments.

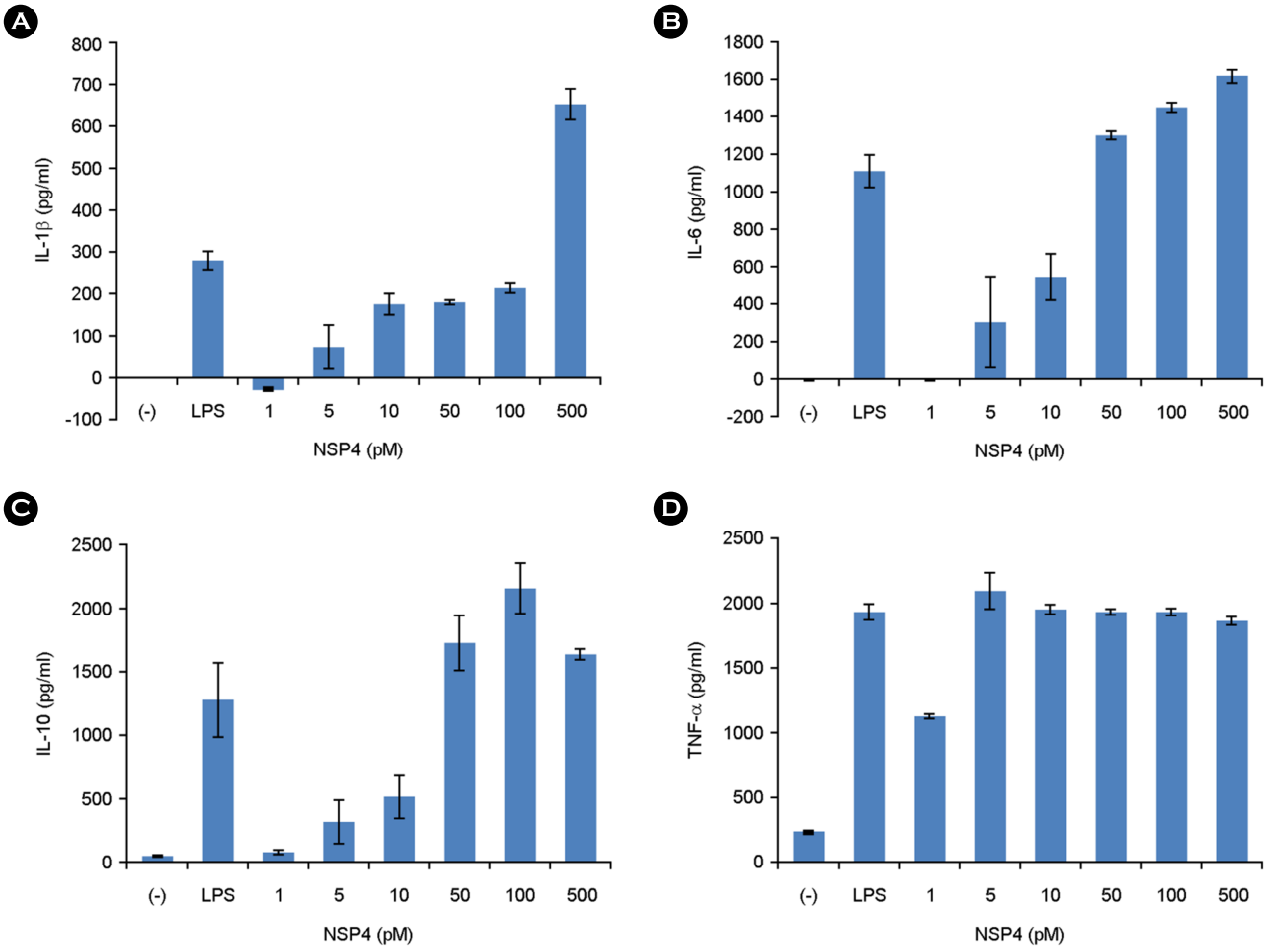

Figure 5. Effect of NSP4 on the levels of IL-1β, IL-6, IL-10, and TNF-α in RAW 264.7 cells. The concentrations of IL-1β, IL-6, IL-10, and TNF-α released into the medium were determined by performing ELISA with the culture supernatant. They were measured as described in the Materials and Methods. Error bars shows the mean ± standard deviation of three measurements.

ml, and 1610 ± 42.13 pg/ml, respectively. Cells exposed to NSP4s had an IL-10 concentration of 74.5 ± 17.69 pg/ml, 313.1 ± 176.13 pg/ml, 508.7 ± 185.51 pg/ml, 1,704.2 ± 231.01 pg/ml, 2134.3 ± 218.33 pg/ml, and 1,620.7 ± 41 pg/ml, and a TNF-α concentration of 1,118.9 ± 21.31 pg/ml, 2,094.9 ± 149.80 pg/ml, 1,940.7 ± 59.71 pg/ml, 1,926.4 ± 32.31 pg/ml, 1,923.4 ± 46.05 pg/ml, and 1,859.5 ± 41.76 pg/ml, respectively (Fig. 5). Significant increase secretion of the IL-1β and IL-6 was at 500-pM of the NSP4 treatment, while the IL-10 was at 100 pM (Fig. 5A, 5B, 5C). In contrast, the secretion of the IL-10 and TNF-α was significantly decline at 500-pM of the NSP4 treatment (Fig. 5C, 5D). These results indicated that the NSP4 caused the occurrence of inflammation in the RAW 264.7 macrophage cells.

DISCUSSION

The rotavirus NSP4 is a well-known viral protein of great interest because of its biological and enterotoxigenic properties. This protein consists of 175 amino acids and contains sequences responsible for membrane-destabilization and enterotoxic effects that have been mapped to different regions of the polypeptide (16). NSP4 is an integral mem-

brane protein residing in the endoplasmic reticulum (ER) with two N-linked oligosaccharide sites at the amino-terminus. This membrane association property makes purification a difficult task (17).

In this study, the rotavirus enterotoxin NSP4 protein was successful expressed and purified in the *E. coli* system. The biological function of the purified NSP4 protein in the inflammatory process was also investigated in the RAW 264.7 macrophage cells. The secretion levels of cytokines, including the IL-1β, IL-6, and TNF-α; and the production of the NO and PGE_2 were significantly increased. These results indicated that NSP4 plays an important role in the inflammatory response observed during rotavirus infection.

E. coli is one of the most widely used expression systems for the production of heterologous proteins because of its well characterized genetic nature and physiology as well as the possibility of strong, inducible expression (18). Because non-glycosylated NSP4 is biologically active, the *E. coli* expression system is preferred in the production of this protein owing to its ability to grow rapidly at high cell density in cultures undertaken using inexpensive substrates (19). By using the strategy discussed in this study, polyhistidine-tagged NSP4 was produced in *E. coli* cells and easily purified with one-step Ni-NTA agarose chromatography under denaturing conditions.

NSP4 stimulates both humoral and cellular immune responses in humans (20). It was also noted that NSP4 elicits systemic and intestinal antibody responses in gnotobiotic pig and rabbit models (17, 21) and induces diarrhea in newborn mice (17, 22). Recently, biliary atresia (BA), a fibro-inflammatory pediatric liver disease that is the primary indication for liver transplantation in children, has been demonstrated to occur in rotavirus infection (3, 23, 24). Osteopontin (OPN), a glycoprotein with inflammatory and fibrogenic activity, may play a pathogenic role in BA (25).

There have been arguments concerning the pathogenesis mechanism of rotavirus virulence components. VP4 is a major determinant in the pathogenesis of BA in the rotavirus-infected newborn mice model (26). NSP4 silencing down-regulates VP7, induces decreased level of nuclear factor-kappa B, and abnormal inactivation of the osteopontin inflammatory pathway in the murine model (27). Despite high bile acid concentration in the liver, the gene expression of canalicular and basolateral hepatobiliary transporters, and their regulatory nuclear receptors was down-regulated with a concomitant increase in the gene expression of inflammatory cytokines as well as in the serum-unconjugated bilirubin content in mice with rhesus rotavirus-induced BA (28).

The mediators secreted by activated macrophages include the superoxide anion and reactive nitrogen intermediates, including NO and cytokines (15). The well-known immune stimulant LPS induces several intracellular signaling pathways in monocytes/macrophages, and effects the production of many inflammatory cytokines and mediators. LPS causes marked pathophysiological reactions via the induction of inflammatory mediators, including NO and PGE_2, and pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6, as well as via the induction of iNOS and COX-2 (9, 29). In response to the immune challenge, macrophages are activated and generate pro-inflammatory mediators that contribute to nonspecific immunity. The role of NSP4 in the inflammatory response mounted against rotavirus infection has not been clearly investigated. Only NSP4 is known to be able to induce iNOS expression and NO production in murine peritoneal macrophages and RAW 264.7 cells (14) as well as the secretion of inflammatory cytokines such as TNF-α, IL-6, and IL-8 (15).

To investigate the hypothesis that during the development of BA, *E. coli*-expressed NSP4 was evaluated by assaying the production of NO, certain pro-inflammatory cytokines (IL-1β, IL-6, and IL-10), and PGE_2. NSP4 stimulated RAW 264.7 cells, causing an increase in the production of NO and PGE_2. In addition, a variety of inflammatory cytokines (IL-1β, IL-6, IL-10, and TNF-α) were shown to be up-regulated in macrophages upon exposure to NSP4. TNF-α is primarily produced by monocytes, macrophages, and T cells (30) and has various pro-inflammatory effects on many cell types.

TNF-α is a potent activator of cells can stimulate the production or expression of IL-1β, IL-6, PGE_2, collagenase, type I and III collagen, and adhesion molecules, and is a

growth factor for both B and T lymphocytes (30, 31). Both TNF-α and IL-1β can lead to cartilage destruction and bone resorption and are important cytokines in chronic inflammatory diseases such as rheumatoid arthritis (30~32). IL-6 plays an important role in many inflammatory conditions and is particularly important in the production of acute phase proteins (33, 34). IL-10 is a pleiotropic cytokine that modulates the function of adaptive immunity-related cells (35). IL-10, although traditionally considered an anti-inflammatory cytokine, has also been implicated in promoting abnormal angiogenesis in the eye and in the pathobiology of autoimmune diseases such as lupus and encephalomyelitis (36).

The LPS inflammatory model has been widely used for understanding and screening of anti-inflammatory effects. However, this system is not enough to study the cellular and molecular mechanisms underlying the inflammation induced by viral infection-related diarrhea and inflammatory diseases. These study findings will be important toward understanding viral gastroenteritis and will provide new avenues for screening, prevention, and treatment of rotavirus. In addition, the NSP4-stimulated RAW 264.7 murine macrophage system could be used as a new inflammatory model system for the evaluation of NO, TNF-α, and various cytokines, namely, IL-1β, IL-6, IL-10, and PGE_2.

In conclusion, our results provide valuable information on the effects of rotavirus NSP4 on the RAW 264.7 macrophage cells, which may play a critical role in the process of both acute and chronic inflammation conditions in rotavirus infection.

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